THE UNIVERSITY OF ALBERTA

A STUDY OF THREE-STRANDED POLYNUCLEOTIDE STRUCTURES

by

© NANCY LEA MURRAY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled A STUDY OF THREE-STRANDED POLYNUCLEOTIDE STRUCTURES submitted by Nancy Lea Murray in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

An attempt has been made to prepare the polypyrimidine RNA's rUC, rUUC, and rUCC for the purpose of testing triplex formation between them and their homologous DNA's dTC:dGA, dTTC:dGAA, and dTCC:dGGA, respectively. E.Coli RNA polymerase was used to synthesize the RNA's from their respective DNA templates. When no interaction between the DNA's and the RNA preparations was detected spectrophotometrically, possible reasons were investigated. Heat denaturation and ethidium bromide binding indicated that the RNA preparations contained secondary structure, possibly in the form of RNA-DNA duplexes. During transcription strand displacement of a DNA strand by the RNA product probably occurred, resulting in the production of the RNA-DNA duplex hybrids. The RNA isolation procedures used would not have resulted in disruption of such a duplex. It is proposed that a subunit or 'factor' of RNA polymerase may normally be involved in DNA unwinding, rewinding, and RNA strand displacement. Our data indicated that such a protein may be storage labile under our conditions.

The properties of the triplex dA:dT:rU were investigated. Mixing curves and band velocity sedimentation indicated that on mixing the polymers dA:dT and poly rU, a single polynucleotide species resulted: the triplex dA:dT:rU. Formation of the triplex resulted in a hypochromic effect between 240 nm and 285 nm.

In the absence of divalent cations, the reaction dA:dT + rU → dA:dT:rU required relatively high monovalent cation concentrations to proceed. The reaction occurred slowly over the KCl or NaCl concentration range of 0.28 M to 0.46 M. However the addition of 1.3 mM $MgCl_2$, in the presence of 5 mM monovalent cations, allowed the reaction to go to completion in a cooperative manner.

Alkaline titration of the triplex showed that the RNA strand melted first, followed by the DNA duplex at a higher pH. The RNA was released from the triplex gradually in the pH interval 8.2 - 9.0.

SDS, an ionic detergent normally used to denature proteins, did not disrupt a preformed triplex, and the three-stranded complex could be formed in the presence of SDS.

Transcription studies of dA:dT:rU indicated that both strands of the DNA template were capable of being transcribed by E.Coli RNA polymerase, though at a much slower rate than dA:dT was transcribed. The presence of the poly rU strand in the triplex caused 65% inhibition of poly rA synthesis, and 80% inhibition of poly rU synthesis. In the presence of 0.2 M KCl, salt conditions thought to exist within an E.Coli bacterium, only 30% inhibition of poly rU synthesis and no inhibition of poly rA synthesis occurred during dA:dT:rU transcription, compared with transcription of dA:dT.

Replication of dA:dT:rU by E.Coli DNA polymerase fraction 7 revealed that its rate was 60% less than the dA:dT replication rate. When M.luteus DNA polymerase was used, the triplex was replicated at a rate 80% slower than the DNA duplex.

Pancreatic ribonuclease degradation of the triplex indicated that the RNA strand was degraded at a rate 30% slower than uncomplexed

RNA. When the triplex was treated with DNase I, the DNA was degraded, although the rate was again 30% slower than that of the uncomplexed DNA.

Equilibrium dialysis studies of spermine binding to dA:dT and dA:dT:rU revealed that the polyamine binds to the triplex at least as well as to the DNA. The fact that spermine binds to DNA in the minor groove supports the supposition that in a DNA-RNA triplex the RNA is wound around the major groove of the DNA.

ACKNOWLEDGEMENTS

I wish to thank Dr. A.R. Morgan for his supervision during the course of these studies.

I would also like to thank Mrs. Terry Elgert for her technical assistance, Mr. Wayne Flintoff, and Mr. Morris Aarbo for his operation of the analytical ultracentrifuge.

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LIST OF ABBREVIATIONS

SDS	sodium dodecyl sulfate
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
$\bar{v}$	partial specific volume
$S^{o}_{20,w}$	intrinsic sedimentation coefficient
Ci	curie
μg	microgram
mg	milligram
μl	microliter (lambda)
DNase	deoxyribonuclease
RNase	ribonuclease
NTP	ribonucleoside triphosphate
dpN	5' deoxyribonucleoside monophosphate
Ap	3' adenosine monophosphate
OD	optical density
u/v	ultraviolet
M	molecular weight
DTT	dithiothreitol
mμmole	millimicromole
λ	wavelength (nanometers)
EDTA	disodium ethylenediamine-tetraacetate

All temperatures are in Centigrade degrees.

CHAPTER 1

SYNTHESIS, ISOLATION, AND CHARACTERIZATION OF POLYPURINE AND POLYPYRIMIDINE RNA'S

INTRODUCTION

Numerous examples of three-stranded polynucleotide structures exist. A list of the triplexes discovered prior to 1967 has been presented in a review article by Felsenfeld and Miles (1).

Triplexes have been observed only in polynucleotides having exclusively either pyrimidines or purines comprising a particular strand. Such asymmetric pieces of DNA have been shown to exist in all bacteriophages, prokaryotes, and eukaryotes studied (2).

Szybalski has presented evidence linking polypyrimidine stretches in DNA with the initiation site for RNA polymerase (2). Miller and Sobell in 1966 presented a model for gene repression which rests on the hypothesis that a gene repressor is a ribonucleoprotein containing an RNA oligonucleotide region which can interact in the deep groove of the DNA helix at the operator locus (3). James Bonner has isolated a unique class of RNA from the chromosomes of higher organisms (4). Called 'chromosomal RNA', it is bound to DNA in an RNase-resistant form, and is covalently bound to chromosomal protein. In pea chromatin these RNA molecules are approximately 40 nucleotides in length, and contain about 27 mole per cent dihydrouridylic acid. Bonner has postulated that these molecules may be involved in the control of

gene expression (5).

The base pairing configurations in triplexes have not yet been established by X-ray crystallography. However, less conclusive evidence suggests that two polynucleotide strands of the triplex are joined by Watson-Crick type base pairing, while the third strand is bonded to only one of the others by either Hoogsteen or reversed Hoogsteen type base pairing (6). These pairing arrangements for T:A:U and C:G:$\overset{+}{\mathrm{C}}$ base pairs are shown in figure 1. In the case where a triplex is formed between one polypurine and two polypyrimidine strands, Hoogsteen pairing between the polypurine and one polypyrimidine strand would imply that the two polypyrimidine strands are antiparallel to one another. Conversely, reversed Hoogsteen base pairing would imply that the two polypyrimidine strands are of parallel polarity.

For the three-stranded polymer rU:rA:rU, the infrared data of Miles suggests a reversed Hoogsteen configuration for the poly rU strand hydrogen bonded to poly rA (7). However, close observation of the reversed Hoogsteen G:C and A:T base pairs reveals that these structures are not isomorphous. For this reason the Hoogsteen type of base pairing would be favored, particularly for polymers in which both G:C and A:T base pairs exist, for example the triplex dTC:dGA:rU$\overset{+}{\mathrm{C}}$. Here the rU$\overset{+}{\mathrm{C}}$ strand is thought to bind to the dGA strand by Hoogsteen base pairing (8).

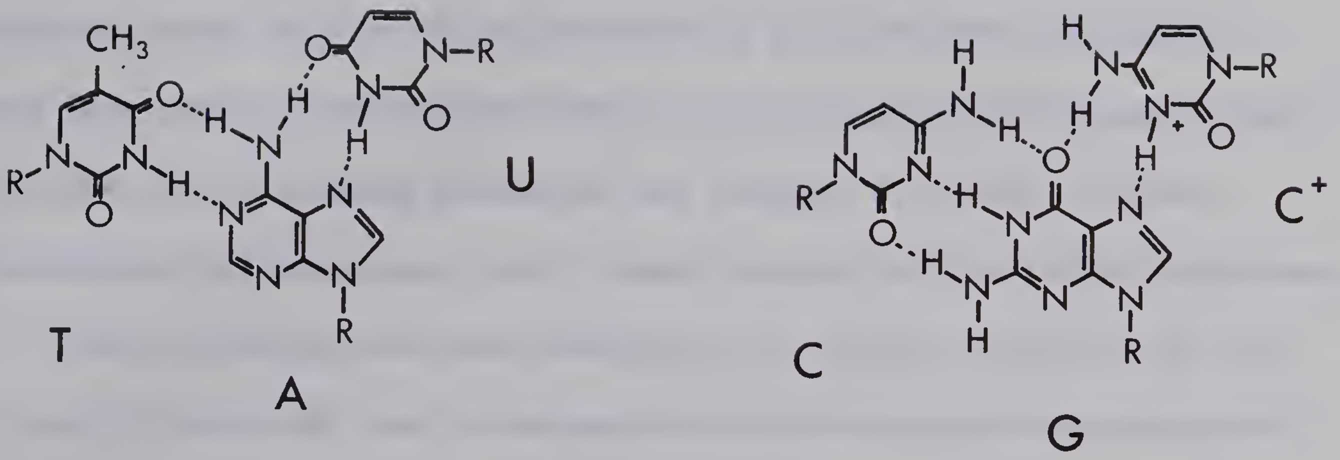
Because of the wide availability of homopolymer DNA's and RNA's, the great majority of triple-stranded structures thus far characterized involve these polymers. However, the existence of a DNA-RNA triplex involving polymers of repeating dinucleotide sequence has been reported

Figure 1. Possible base pairing arrangements in triplexes.

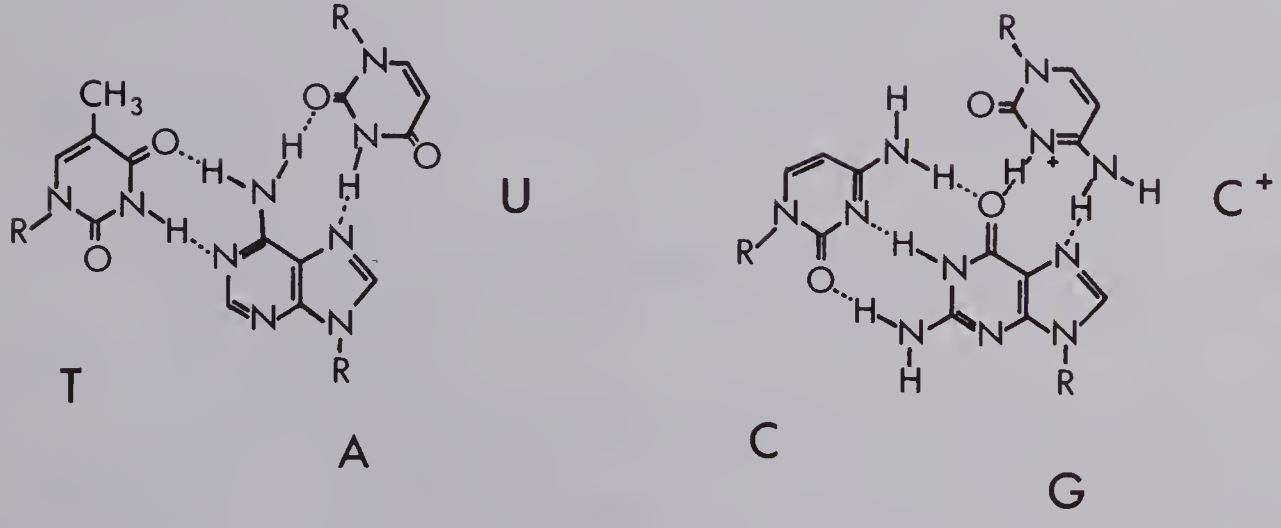
(a) Watson-Crick and Hoogsteen base pairing;

(b) Watson-Crick and reversed Hoogsteen base pairing.

(a)



(b)



by Morgan and Wells (8). The triplex, dTC:dGA:rU$\overset{+}{C}$, exists in acidic solution, where the cytosine in poly rUC is protonated. The formation of a DNA-RNA triplex involving dTC:dGA shows a specificity dependent on the sequence of bases in RNA. Thus poly rG, poly rC, poly rU, poly rA, and poly rGA did not interact with dTC:dGA. The random copolymer poly r(U,C)(1:1.25) interacted with dTC:dGA, although a much less stable complex was formed than that of poly rUC and dTC:dGA. The poly r(U,C) complex formation was thought to be due to short sequences of alternating C and U bases present in the random copolymer.

The following work was undertaken to further characterize the triplex dTC:dGA:rU$\overset{+}{C}$, and to extend the data on sequence specificity of triplex formation to polynucleotides containing a repeating trinucleotide sequence.

MATERIALS AND METHODS

E.Coli B, 3/4 log phase cells were purchased from Grain Processing Co. as a frozen paste.

^{14}C-ribonucleoside and deoxyribonucleoside triphosphates were purchased from Schwarz BioResearch, Inc. Unlabeled triphosphates were purchased from P-L Biochemicals. Omnifluor scintillation fluor was obtained from New England Nuclear (Canada) Ltd.

Sigma Chemicals supplied the ethidium bromide, and Bio-Rad Laboratories the Biogel agarose beads.

The four nucleases, spleen phosphodiesterase, micrococcal nuclease, snake venom phosphodiesterase, and deoxyribonuclease I (specific activity 22 units/mg) were purchased from Worthington Biochemical Corporation. The DNase I as supplied was electrophoretically purified, and consequently RNase-free by their hyperchromicity assay.

The synthetic DNA's, dTC:dGA, dTG:dCA, dTTC:dGAA, and dTCC:dGGA were prepared from oligonucleotides which originated in Dr. H.G. Khorana's laboratory.

All spectrophotometric measurements were made using a Gilford 2400 spectrophotometer.

E.Coli RNA polymerase was prepared according to the method of Chamberlain and Berg (9), except for the following modifications:

1) Centrifugation of the crude extract was lengthened to 12 hours at 30,000 rpm in the #30 Spinco rotor;
2) DEAE-cellulose chromatography was replaced by Biogel A-1.5m (200-400 mesh) column chromatography;
3) A final column chromatography on Biogel A-0.5m (200-400 mesh) was

carried out.

The specific activity of various enzyme preparations ranged from 1800 to 3000 units/mg (1 unit corresponds to the incorporation of 1 mμmole of CMP per hour under the conditions described below, and using calf thymus DNA as template).

Synthesis of RNA was followed by the incorporation of ^{14}C-ribonucleoside triphosphates into acid insoluble polynucleotide. A standard assay mixture contained in 0.10 ml:

50 mM tris pH 8
5 mM $MgCl_2$
10 mM mercaptoethanol
0.5 mM of each NTP (one ^{14}C-labeled, specific activity 5 Ci/mole)
30 - 90 mμmoles/ml DNA
0.16 - 0.80 mg/ml E.Coli RNA polymerase

While incubating the reaction mixture at 37°, aliquots were removed at appropriate times and spotted on filter papers. Subsequently the filters were washed in cold 5% TCA by swirling gently on a rotary shaker for 15 minutes. After 3 similar washes, followed by two 5 minute washes in 95% ethanol, the filters were oven-dried and counted in a Beckman LS-250 scintillation counter. The scintillation fluid used contained 14.4 g Omnifluor in 3.8 liters of toluene.

Preparative RNA synthesis was carried out by scaling up the above assay mixtures to an appropriate volume, and providing only unlabeled NTP's as substrates. After incubating at 37° for a predetermined length of time, the reaction mixture was made 0.01% in SDS and 10 mM in EDTA before concentrating. Concentration was carried out by placing the sample in dialysis tubing and surrounding the tubing with polyethylene glycol. The reaction mixture was then applied to a Biogel A-1.5m (200-400 mesh) column equilibrated with 10 mM tris pH 8,

0.1 mM EDTA. The DNA and RNA eluted in the void volume, while remaining NTP's and RNA polymerase appeared in an included peak. After concentration of the fractions containing DNA and RNA on an Evapo-mix rotary evaporator, the solution was made 2 M with respect to KCl, and applied to a Biogel A-15m (200-400 mesh) column equilibrated with buffer containing 2 M KCl, 10 mM tris pH 8, 0.1 mM EDTA. The DNA was eluted in the void volume, while the RNA was partially included. After concentration of the RNA, it was dialyzed against 1000 volumes of 0.5 M KCl, 10 mM tris pH 8, 0.1 mM EDTA, and finally against two changes of 1000 volumes of 10 mM tris pH 8, 0.1 mM EDTA. It was stored at -20°. In some cases the RNA was isolated by an alternate procedure. After incubating the reaction mixture at 37° for an appropriate length of time, DNase I was added to a final concentration of 10 μg/ml. After a further incubation at 37° for one hour, the reaction was stopped by adding SDS and EDTA to final concentrations of 0.01% and 0.1 mM respectively. The sample was then concentrated and applied to a Biogel A-1.5m (200-400 mesh) column equilibrated with 10 mM tris pH 8, 0.1 mM EDTA. The RNA was eluted in the excluded peak, separated cleanly from the DNase I, RNA polymerase, NTP's, and dpN's. The RNA was then concentrated and dialyzed as described above.

Sedimentation measurements were made in a Spinco model E ultracentrifuge equipped with u/v optics. Band sedimentation was performed using a Vinograd 4-12 quartz cell. 20 μl of DNA at a concentration of 2 OD/ml was layered onto a solvent and centrifuged at 56,000 rpm and 25°. For a sedimentation carried out under neutral pH conditions, the solvent contained 1 M NaCl, 50 mM Na phosphate pH 6.7, while the DNA sample contained 50 mM Na phosphate pH 6.7. The solvent

used for alkaline sedimentation contained 0.9 M NaCl, 0.1 M NaOH. In this case the DNA sample was made up in 0.1 M NaOH.

E.Coli DNA polymerase fraction 7 was prepared by Dr. A.R. Morgan according to the method of Kornberg (10, 11). E.Coli nuclease fraction DIII (prepared by Mr. Wayne Flintoff) was obtained from a crude fraction of the RNA polymerase purification procedure. After extracting the protamine sulfate pellet with buffer containing 0.1 M $MgCl_2$, 10 mM tris pH 8, and 10 mM mercaptoethanol, ammonium sulfate was added to the supernatent to a final concentration of 65%. The resulting precipitate was dissolved in buffer containing 5 mM $MgCl_2$, to a final concentration of 5 mg/ml protein.

^{32}P-labeled E.Coli DNA was prepared by Dr. A.R. Morgan. The final step in its purification was banding in a CsCl density gradient.

^{14}C-labeled dTG:dC*A was prepared by incubating E.Coli DNA polymerase fraction 7 with dTG:dCA in the presence of the four deoxyribonucleoside triphosphates, the dCTP being ^{14}C-labeled with a specific activity of 1 Ci/mole. The reaction mixture contained:

50 mM Na phosphate pH 7.5
15 mM $MgCl_2$
1 mM in each dNTP
93 mμmoles/ml dTG:dCA
40 μg/ml E.Coli DNA polymerase fraction 7
25 μg/ml fraction DIII

After a 4 hour incubation at 37°, the reaction was stopped by adding SDS and EDTA to a final concentration of 0.01% and 30 mM respectively. The DNA was isolated in the excluded peak of Biogel A-0.5m (200-400 mesh) column chromatography.

DNA molar extinction coefficients were determined by observing their hyperchromicity after addition of either:

1) micrococcal nuclease, followed by spleen phosphodiesterase;

or 2) DNase I, followed by snake venom phosphodiesterase. Molar extinction coefficients of RNA's were determined by observing their hyperchromicity after addition of micrococcal nuclease, followed by spleen phosphodiesterase. A micrococcal nuclease - spleen phosphodiesterase digestion was carried out by mixing 30 - 70 mμmoles/ml of DNA or RNA with 10 mM glycine buffer pH 9.2 in a cuvette at 25°. After reading the absorbance at 260 nm and λ_{max}, the temperature was raised to 37° in the cell compartment of the spectrophotometer by means of a water circulator. $CaCl_2$ was added to a final concentration of 2 mM and micrococcal nuclease to a final concentration of 0.25 μg/ml. The optical density at 260 nm was followed with time until no further change was observed. The solution was then made 100 mM in NH_4 acetate buffer pH 5.9, and 50 μg/ml in spleen phosphodiesterase. Absorbancy at 260 nm was followed until no further change was observed. In all cases absorbance was measured using a blank having the same composition as the sample, but lacking nucleic acid.

DNase I and snake venom phosphodiesterase digestions of DNA were carried out similarly. However, the initial absorbance of 30 - 70 mμmoles/ml of DNA in 5 mM NaCl, 5 mM tris pH 8 was recorded. After heating to 37°, the sample was made 2 mM in $MgCl_2$ and 3 μg/ml in DNase I. When the absorbance reached a plateau value, the solution was made 100 mM in NH_4 acetate buffer pH 9, 10 mM in $MgCl_2$, and 50 μg/ml in snake venom phosphodiesterase. The nucleotide concentrations were determined by reference to known extinction coefficients (Schwarz BioResearch Radiochemical Catalogue).

High voltage electrophoresis was carried out using a lead plate, electrode assembly, and power source manufactured by Savant Instruments,

Inc. Approximately 1 OD unit of nucleotide was spotted on Whatman 3 MM paper and dried. Samples containing divalent cations, or greater than 0.05 M monovalent cations were first passed over a Dowex 50 pyridinium column (0.6 X 4.0 cm) and eluted with water. After concentrating the sample on an Evapo-mix rotary evaporator, it was spotted and dried. The entire strip was then moistened with electrophoresis buffer (0.85 M NH_4 acetate pH 3.5, 0.1 mM EDTA), and subsequently electrophoresed at 2000 volts for 2 hours. The operating temperature was maintained at 10 - 15°. After air drying the paper, the samples were visualized by irradiating with a u/v lamp. Radioactive compounds were detected by cutting the paper into 1 X 3 cm strips and counting each in toluene-based scintillation fluid.

Absorption spectra were measured by changing the wavelength manually and taking individual readings at each wavelength. The Gilford specifications state an accuracy of ±0.5 nm.

Fluorometry was carried out using a G.K. Turner Model 210 spectrofluorometer. Approximately 0.01 OD unit of nucleic acid in 2 - 25 μl was added to 2 ml of a solution containing 0.5 μg/ml ethidium bromide, 2 mM tris pH 8.5, 0.2 mM EDTA. The solution was excited at 525 nm, and intensity of fluorescence emission at 600 nm was measured.

RESULTS

Determination of Optimal Conditions for Polypurine and Polypyrimidine RNA Synthesis

The polypurine RNA's rGA, rGGA, and rGAA showed similar kinetics of synthesis. Analogously, the kinetics of formation of the polypyrimidine RNA's rUC, rUUC, and rUCC were similar. Representative kinetic patterns for rUCC and rGGA synthesis are shown in figure 2. These plots were obtained in the absence of KCl. When KCl was added to a reaction mixture, there was a stimulation of polypurine synthesis, and an inhibition of polypyrimidine synthesis. The optimal KCl concentration for stimulation of polypurine RNA synthesis was found to be 0.2 M. These results are shown in figures 3 and 4 for poly rUCC and poly rGGA synthesis. These results are similar to those reported by Morgan (12). However, he did not observe a plateauing of polypyrimidine RNA synthesis, but rather an initial burst followed by a linear phase of synthesis which exceeded polypurine synthesis after between 1 and 2 hours.

For both polypurine and polypyrimidine RNA synthesis, the optimal nucleotide triphosphate concentration was determined to be 0.5 mM for each NTP for transcription of dTC:dGA. The optimal NTP concentrations for transcription of DNA's with repeating trinucleotide sequences was 0.5 mM and 1.0 mM for the unique and repeated base, respectively.

That the RNA products of in vitro transcription contained the expected stoichiometry of bases was confirmed by measuring the incorporation of each base present in the RNA with time, under

Figure 2. Synthesis of poly rUCC and poly rGGA with time. The reaction mixtures contained 50 mM tris pH 8, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5 mM UTP or ATP, 1.0 mM ^{14}C-CTP or ^{14}C-GTP, 50 mμmoles/ml dTCC:dGGA, 0.35 mg/ml *E.Coli* RNA polymerase. Synthesis was carried out at 37°.

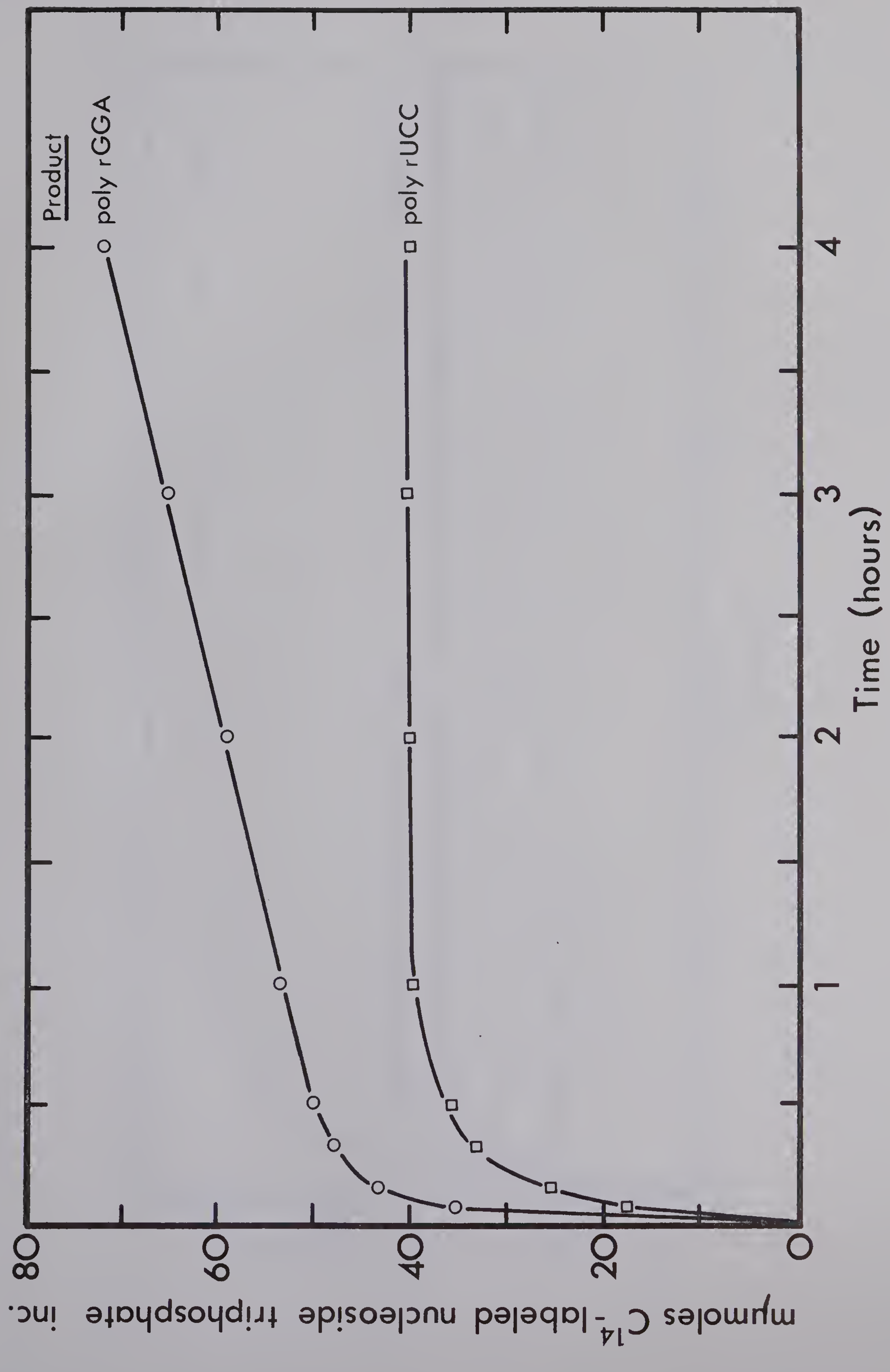

mμmoles C14-labeled nucleoside triphosphate inc.
0
20
40
60
80
1
2
3
4
Time (hours)
Product
poly rGGA
poly rUCC

Figure 3. Synthesis of poly rUCC as a function of KCl concentration. The reaction mixtures contained 50 mM tris pH 8, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5 mM UTP, 1.0 mM ^{14}C-CTP, 50 mμmoles/ml dTCC:dGGA, 0.35 mg/ml *E.Coli* RNA polymerase, and appropriate amounts of KCl. Synthesis was at 37°.

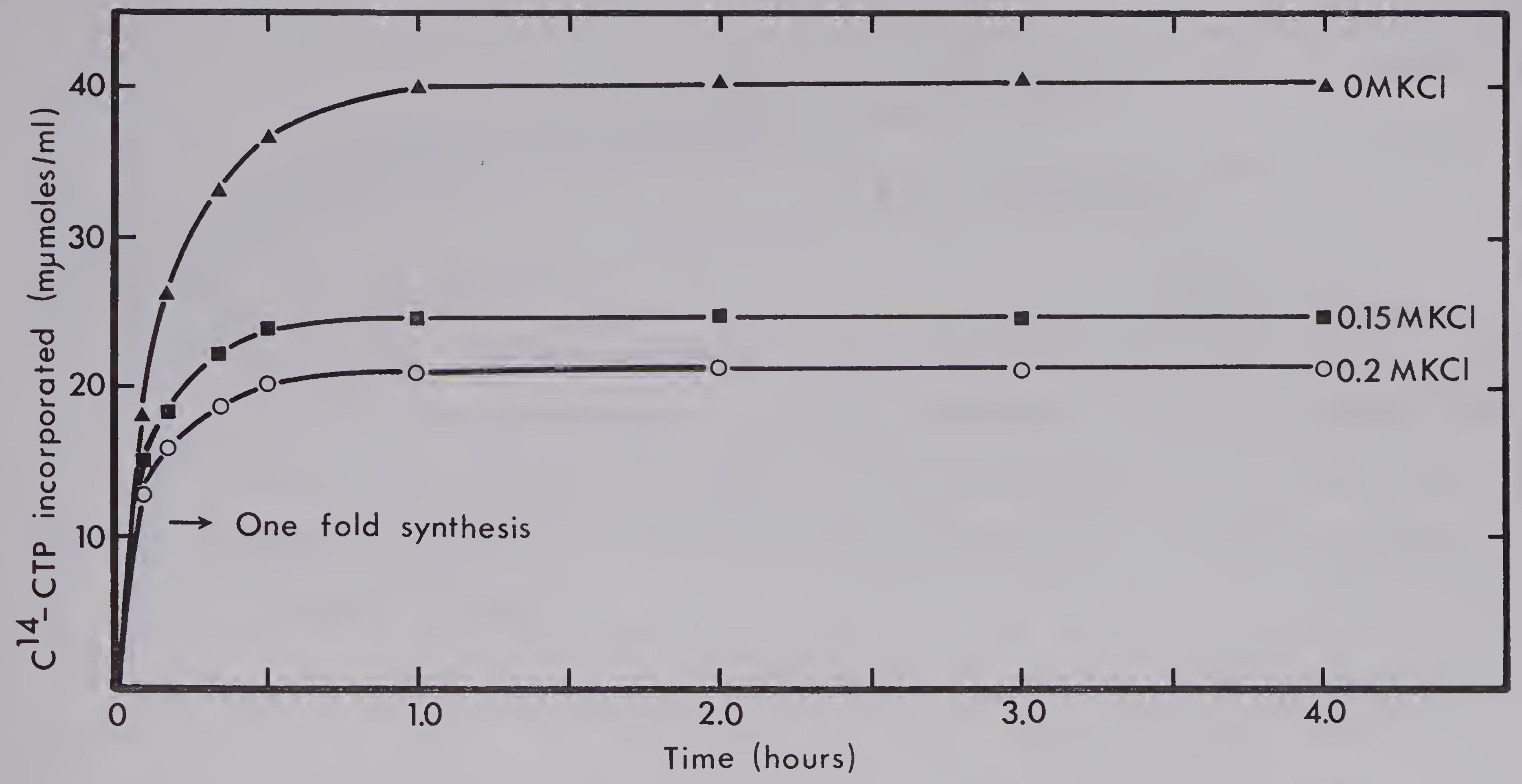
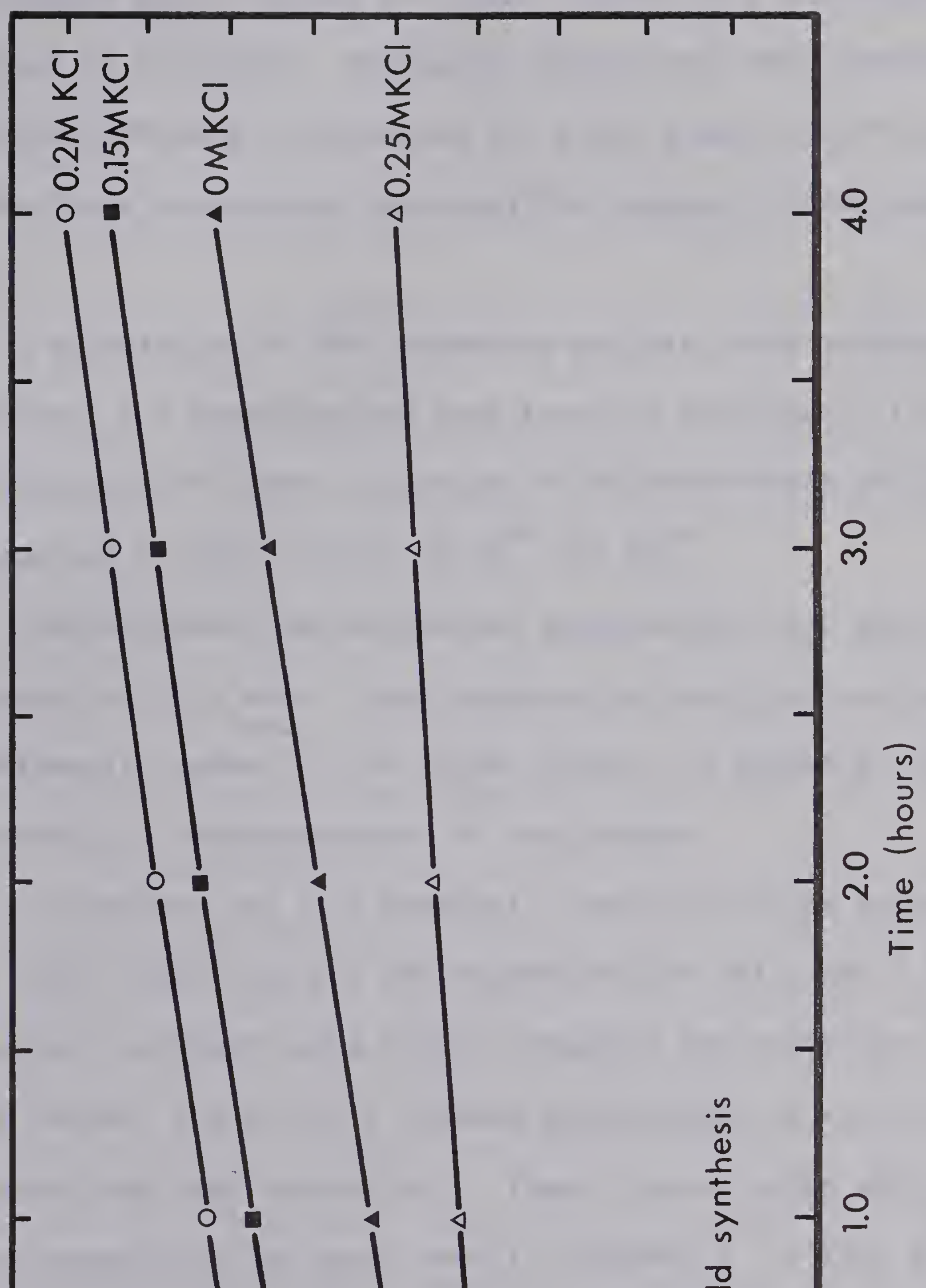

C^{14}- CTP incorporated (mμmoles/ml)
40
30
20
10
0
1.0
2.0
3.0
4.0
Time (hours)
▲ 0MKCl
■ 0.15 MKCl
○ 0.2 MKCl
→ One fold synthesis

Figure 4. Synthesis of poly rGGA as a function of KCl concentration. The reaction mixtures contained 50 mM tris pH 8, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5 mM ATP, 1.0 mM ^{14}C-GTP, 50 mμmoles/ml dTCC:dGGA, 0.35 mg/ml *E.Coli* RNA polymerase, and appropriate amounts of KCl. Synthesis was at 37^{o}.


0.2M KCl
0.15MKCl
0MKCl
0.25MKCl
→ One fold synthesis
C14-GTP incorporated (mμmoles/ml)
20
40
60
80
0
1.0
2.0
3.0
4.0
Time (hours)

identical conditions. For example, the amounts of ^{14}C-CTP and ^{14}C-UTP incorporated into ^{14}C-poly rUC* and ^{14}C-poly rU*C respectively were identical at all times. Similarly, during poly rUUC synthesis, 2 moles of UTP were incorporated for every 1 mole of CTP incorporated. All the RNA's synthesized contained the expected stoichiometry of bases.

A stimulation of RNA polymerase activity when manganese was substituted for magnesium has been reported previously (13). However, no stimulation of either polypurine or polypyrimidine RNA synthesis was observed on substitution of Mn^{+2} for Mg^{+2}.

Five different RNA polymerase preparations were used throughout the course of this work. Each preparation contained approximately a stoichiometric amount of the sigma subunit, as judged by SDS polyacrylamide gel electrophoresis of the enzymes.

The maximum net fold synthesis observed for any polypurine RNA was 9 fold, while that for the polypyrimidine RNA's was 5 fold. These values were obtained using freshly prepared RNA polymerase. On storage of the enzyme, the activity dropped substantially during the first two weeks, and then leveled off. Thus, a preparation which had been stored longer than two weeks usually provided 3 - 4 fold net synthesis for a polypurine RNA, and 1 - 2 fold net synthesis for a polypyrimidine RNA.

Investigation of the Reasons for Poor Net Fold Transcription

In an attempt to gain insight into possible reasons for the poor net synthesis of RNA, several ideas were tested:

1) Limiting NTP substrate concentration was ruled out as a reason for poor RNA synthesis. For both polypurine and polypyrimidine RNA synthesis, a maximum of 10% of the NTP's supplied was used before synthesis plateaued. If greater concentrations of NTP's and $MgCl_2$ were supplied (extra $MgCl_2$ was added to compensate for NTP - Mg^{+2} complexing), no stimulation of either initial rates of synthesis or net synthesis was achieved.
2) If the RNA polymerase were becoming inactivated during the course of the reaction, then plateauing of RNA synthesis could be explained by postulating the absence of active RNA polymerase. To test this hypothesis, a reaction mixture containing a saturating concentration of RNA polymerase was allowed to synthesize RNA until a plateau was reached. Additional RNA polymerase was added at this point, and further RNA synthesis was followed with time. In fact, no newly synthesized material could be detected from that point on. The conclusion was that RNA polymerase inactivation was not responsible for plateauing of RNA synthesis.
3) If the limiting factor in RNA synthesis were reinitiation by RNA polymerase after having completed an RNA molecule, then the size of the DNA template would affect the net synthesis achieved. For this reason, band sedimentation experiments were carried out on the dTC:dGA template. Calculations were done using the relationships established by Studier (14):

$$\text{neutral } S^{o}_{20,w} = 1.144\ s = 0.0882\ M^{0.346}$$

$$\text{alkaline } S^{o}_{20,w} = 1.160\ s = 0.0528\ M^{o.40}$$

The results are shown below, along with the values reported by Wells and Blair for dTC:dGA (15). It was with their preparation of dTC:dGA template that poly rUC net fold synthesis as high as 10 could be achieved (Dr. A.R. Morgan, personal communication).

	ALKALI		NEUTRAL	
	$S^{o}_{20,w}$	$M \times 10^{-5}$	$S^{o}_{20,w}$	$M \times 10^{-5}$
dTC:dGA (Wells & Blair (15))	8.13	2.92	8.85	6.08
dTC:dGA	5.40	1.10	7.22	3.40

These results indicated that our DNA was approximately one half the molecular weight of the DNA of Wells and Blair, and contained more single strand nicks. RNA polymerase is thought to bind to single strand nicks and initiate RNA synthesis from that point. The observed reduction in molecular weight and small increase in the number of single strand nicks in our template would not be expected to be sufficient to cause such a great reduction in net synthesis as was observed.

4) Both initial rates of RNA synthesis and net synthesis attainable decreased rapidly on storage of the enzyme. The most rapid decay of enzyme activity occurred within the first two weeks of storage, after which the activity remained constant. Two methods of storage of the enzyme were tested:

   a) frozen at -20° in buffer containing 0.1 mM DTT, 10 mM tris

pH 8, 1 mM $MgCl_2$, 5% glycerol (v/v);

b) in solution at -20^o in buffer containing 0.1 mM DTT, 10 mM tris pH 8, 1 mM $MgCl_2$, 50% glycerol (v/v).

Neither method of storage avoided the initial rapid decay of enzyme activity.

Preparative RNA Synthesis and Isolation

Polypurine and polypyrimidine RNA's were synthesized in relatively large quantities by scaling up appropriate assay mixtures.

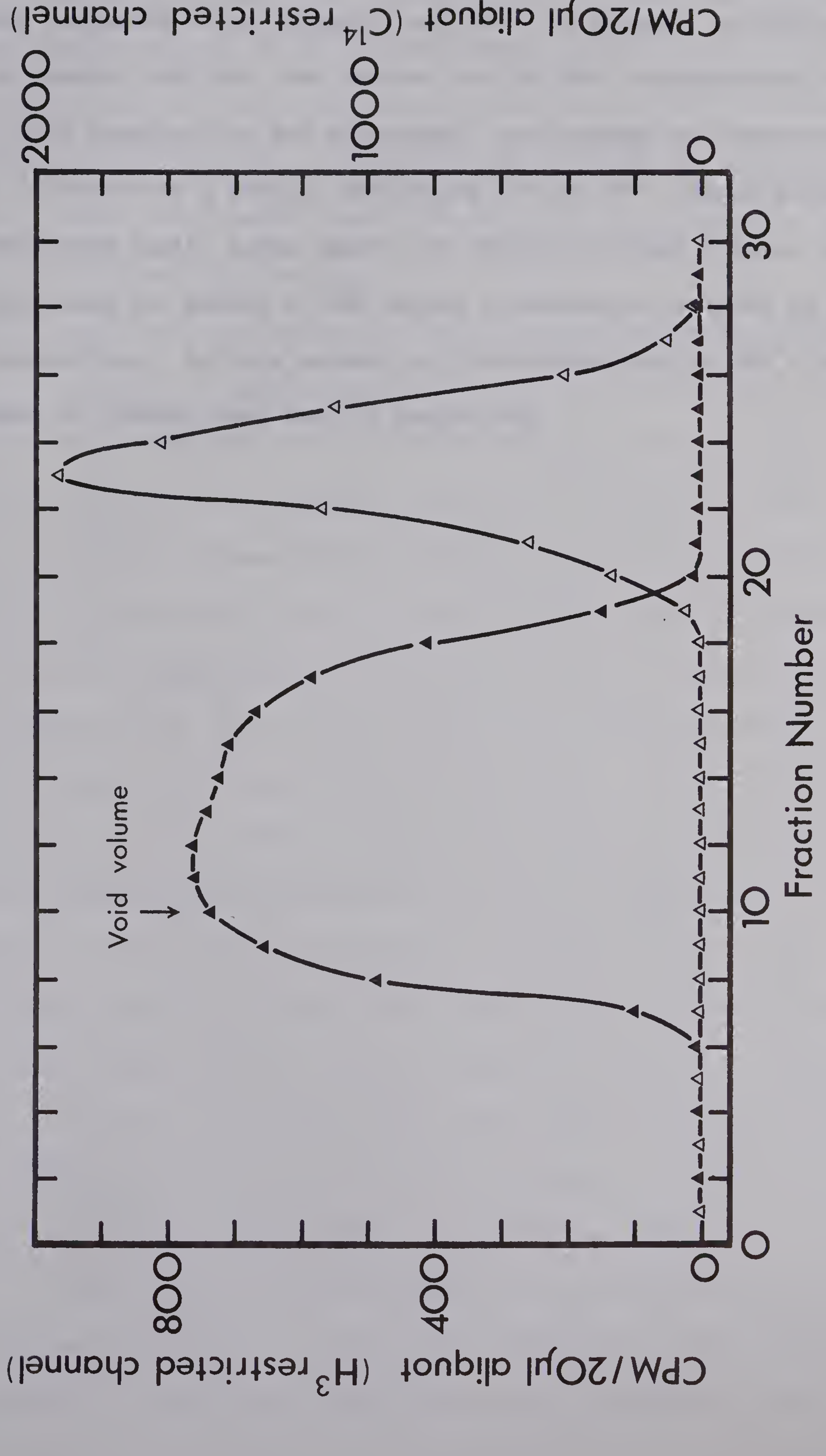
Polypurine RNA's were isolated by passage over two Biogel columns. The first column was a Biogel A-1.5m (200-400 mesh) column equilibrated with buffer of low ionic strength. The DNA template and RNA product were eluted in a void volume peak. After concentration of this void volume material, it was passed over a Biogel A-15m (200-400 mesh) column equilibrated with buffer of high ionic strength. Two peaks of polynucleotide were eluted from this column. The void volume peak was DNA, while the included peak was newly synthesized RNA.

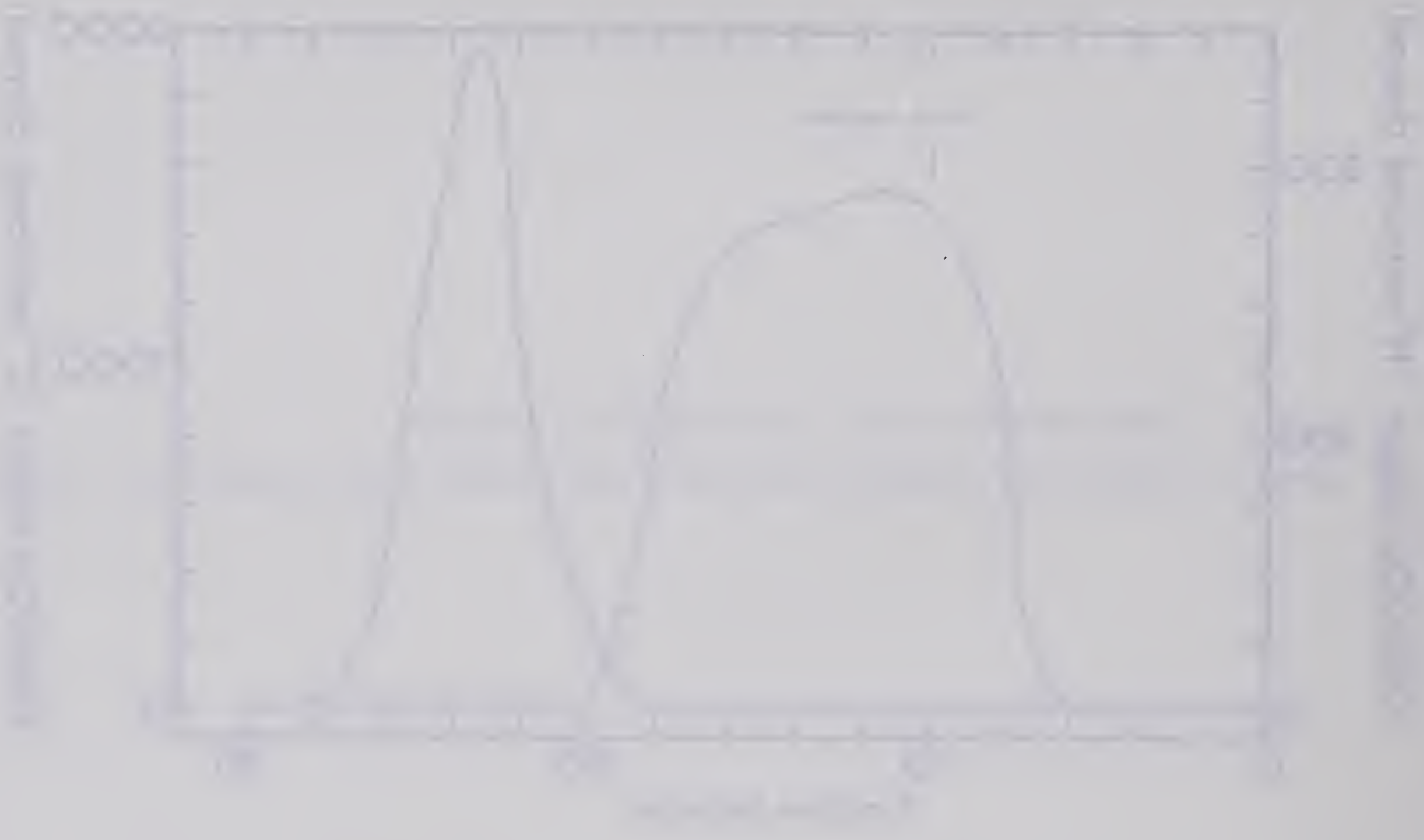
The separation of DNA from RNA on Biogel A-15m in high salt was demonstrated using a sample containing ^{3}H-labeled dTG:dC*A and ^{14}C-labeled poly rUG*. The results are shown in figure 5. The DNA appeared to be rather heterogeneous with respect to size. Nonetheless, the DNA and RNA were well separated by this column chromatography.

The RNA eluted from a Biogel A-15m column was checked for DNA contamination by an RNA polymerase assay. For example, to test for contamination of poly rGA by dTC:dGA, the poly rGA was supplied as a template in an RNA polymerase reaction, and incorporation of ^{14}C-ATP into a polymer containing ATP and GTP was tested. In every case the polypurine RNA's were shown to be free of contaminating DNA.

When polypyrimidine RNA's and their template DNA's were passed over Biogel A-15m in high salt, often poor resolution of the two species was obtained. For this reason, these RNA's were isolated by treating the original reaction mixture with DNase I after completion of the RNA synthesizing reaction. The RNA was then isolated in the void

Figure 5. Separation of ^{3}H-dTG:dC*A and ^{14}C-poly rUG* on Biogel A-15m (200-400 mesh). Elution buffer contained 2 M KCl, 10 mM tris pH 8, 0.1 mM EDTA.


CPM/20μl aliquot (C^{14} restricted channel)
2000
1000
0
CPM/20μl aliquot (H^3 restricted channel)
800
400
0
0
10
20
30
Fraction Number
Void volume



volume peak of a Biogel A-1.5m column chromatography. The DNase I, RNA polymerase, NTP's, and 5' deoxyribonucleoside monophosphates were contained in an included peak from the column. An RNA so isolated, for example poly rUC, was checked for dTC:dGA contamination by providing it as a template for RNA polymerase, and looking for incorporation of ^{14}C-UTP into a product containing UTP and CTP. Assay mixtures containing small, known amounts of dTC:dGA provided a basis for estimating the amount of DNA duplex contamination present in the RNA preparations. By this method, all the polypyrimidine RNA's were shown to contain less than 5% duplex DNA.

Molar Extinction Coefficient Determinations

The molar extinction coefficients of the polypurine-polypyrimidine DNA's were obtained by degrading them completely with nucleases, and determining the nucleotide concentrations by reference to known extinction coefficients. The DNA's were degraded by two methods:

1) DNase I and snake venom phosphodiesterase digestion, yielding 5' mononucleotides, and
2) micrococcal nuclease and spleen phosphodiesterase digestion, yielding 3' mononucleotides.

The results obtained from both methods agreed within 1%. Representative plots obtained from enzymatic digestions of dTC:dGA are shown in figure 6.

To ensure that complete degradation of the DNA to mononucleotides was being achieved, similar reaction mixtures were prepared using ^{32}P-labeled <u>E.Coli</u> DNA as a substrate. When the hyperchromicity reached a plateau value, the reaction mixtures were freed of divalent cations by passage over a Dowex 50 pyridinium column, and then electrophoresed on paper in pH 3.5 buffer. The results of a micrococcal nuclease and spleen phosphodiesterase digestion of ^{32}P-<u>E.Coli</u> DNA are shown in figure 7. The positions of the four unlabeled ribonucleoside monophosphate markers are also shown. The ribo- and deoxyribo-mononucleotides have the same mobilities in this separation procedure. Electrophoresis of the products of a DNase I and snake venom phosphodiesterase digestion of ^{32}P-<u>E.Coli</u> DNA also indicated that complete digestion to mononucleotides had occurred under the conditions used.

Molar extinction coefficients of the polypurine and polypyrimidine RNA's were determined by micrococcal nuclease and spleen phosphodiesterase digestion. That complete degradation was being achieved was confirmed

Figure 6. Enzymatic degradation of dTC:dGA using 1) micrococcal nuclease and spleen phosphodiesterase, and 2) DNase I and snake venom phosphodiesterase. The micrococcal nuclease digestion mixture contained 60.4 mμmoles/ml dTC:dGA, 10 mM glycine buffer pH 9.2, 2 mM $CaCl_2$, and 0.25 μg/ml micrococcal nuclease. When the absorbance at 260 nm reached a plateau, the digest was made 100 mM in NH_4 acetate buffer pH 5.9, and 50 μg/ml in spleen phosphodiesterase.

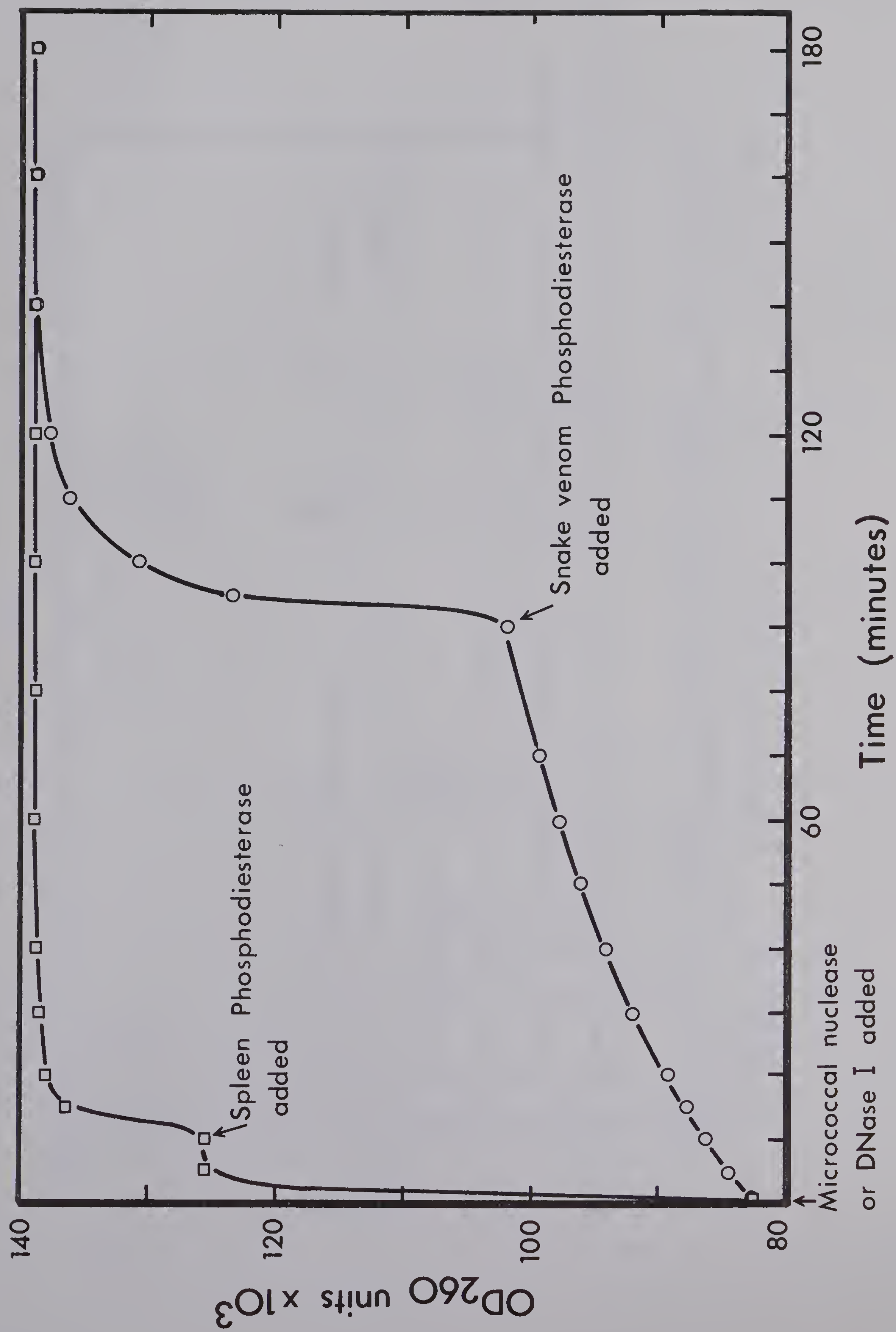
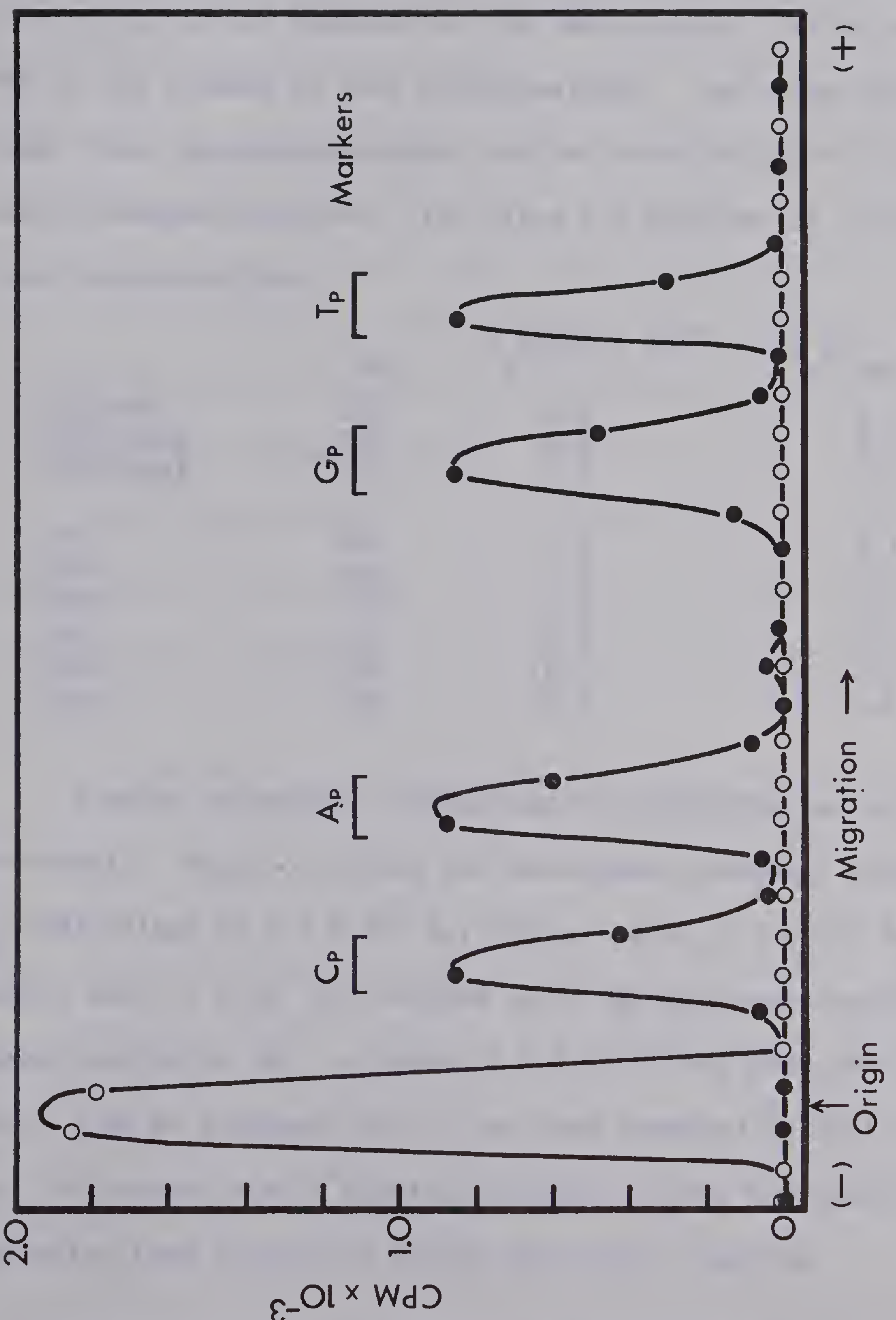

OD$_{260}$ units x 10^3
140
120
100
80
Time (minutes)
60
120
180
Spleen Phosphodiesterase added
Snake venom Phosphodiesterase added
Micrococcal nuclease or DNase I added

Figure 7. Paper electrophoresis of ^{32}P-*E.Coli* DNA before and after digestion by micrococcal nuclease and spleen phosphodiesterase. The electrophoresis buffer contained 0.85 M NH_4 acetate pH 3.5, 0.1 mM EDTA. Electrophoresis was carried out at 10 - 15° and 2000 volts for 2 hours.

○——○ undigested ^{32}P-*E.Coli* DNA; ●——● digested ^{32}P-*E.Coli* DNA

The marker positions are shown.


CPM × 10⁻³
2.0
1.0
0
Markers
Tp
Gp
Ap
Cp
(+)
(−)
Origin
Migration →

by electrophoresing the products of a digestion mixture containing ^{14}C-labeled poly rA*U* as substrate. After digestion all the radioactivity was transferred from the origin to two spots corresponding to Ap and Up.

The results of the DNA and RNA molar extinction coefficient determinations are compiled in the table below. The value for each DNA is the average of four determinations: two using DNase I and snake venom phosphodiesterase, and two using micrococcal nuclease and spleen phosphodiesterase. The value for each RNA is the average of three determinations.

	λ_{max}	$\varepsilon_m(260) \times 10^{-3}$	$\varepsilon_m(\lambda_{max}) \times 10^{-3}$
dTC:dGA	258	6.5	6.6
dTCC:dGGA	255	6.8	6.9
dTTC:dGAA	255	6.3	6.4
* rUC	254	5.1	5.3
* rUCC	255	5.2	5.3
* rUUC	259	5.2	5.3
rGA	257	8.8	9.0
rGGA	255	10.2	10.4
rGAA	255	7.5	7.8

A molar extinction coefficient for dTCC:dGGA has not been reported previously. Based on ashing and subsequent phosphate determinations, $\varepsilon_m(260)$ values of 6.7×10^3 for rUC in water, 7.8×10^3 for rGA in water, and 5.7×10^3 for dTC:dGA in 10 mM NaCl were reported by Morgan and Wells (8). A value of 5.2×10^3 for dTTC:dGAA in 0.2 M NaCl, 2 mM Na phosphate pH 7.3 has been reported by Wells et al. (16). The difference in salt conditions would account for some of the variation from literature values previously reported.

* These preparations are likely polypyrimidine RNA - polypurine DNA duplexes (see discussion), and hence the values determined are invalid.

Absorption Spectra of DNA, RNA, and DNA-RNA Mixtures

Absorption spectra of dTC:dGA and poly rUC were determined in buffer containing 1 M NaCl, 10 mM Na phosphate pH 5.8, 5 mM $MgCl_2$. The DNA and RNA were then combined in a phosphate molar ratio of 2:1, and the absorption spectrum of the mixture was determined. No hypochromicity was observed at any wavelength between 225 nm and 295 nm, relative to a calculated spectrum for a non-interacting mixture of dTC:dGA and poly rUC in that ratio. Previously a triplex has been shown to form under these buffer conditions, resulting in a hypochromic effect (8). Similarly, no interaction could be shown to exist between dTC:dGA and poly rUC when the pH was lowered to 4.5, or the NaCl concentration reduced to 100 mM.

These same experiments were performed with dTCC:dGGA and dTTC:dGAA and their corresponding RNA's, poly rUCC and rUUC, respectively. No DNA-RNA interaction was observed for either system under any of the three buffer conditions.

Tests for Double-Strandedness of Polypyrimidine RNA's

In an attempt to rationalize the irreproducibility of dTC:dGA and poly rUC interaction, several possibilities were considered. It was decided that the most likely source of difficulty was the RNA. If some type of secondary structure were to exist in the RNA, then its interaction with DNA might be prevented. To test this hypothesis, poly rUC was heated to 100^{o} in buffer containing 2 mM tris pH 8.5, 0.2 mM EDTA, and then quick cooled on ice. Under these conditions, a polymer which is not self-complementary will not renature immediately. The poly rUC preparation showed approximately 20% hyperchromicity by this test. When a final concentration of 5 mM $MgCl_2$ was added to the heated and quick-cooled sample, a return to the original absorbance was observed.

Further evidence for secondary structure came from spectrofluorometric studies of the poly rUC, poly rUUC, and poly rUCC preparations. It has been shown by LePecq and Paoletti that on binding of ethidium bromide to polynucleotides, a fluorescent complex having a strikingly enhanced fluorescence quantum yield is formed (17). This increase in fluorescence is a specific test for double stranded polynucleotide structures. When ethidium bromide was added to any of the polypyrimidine RNA preparations, an increase in the fluorescence efficiency was observed. This enhanced fluorescence could be reduced almost to background level by heating and quick cooling the RNA preparations in the presence of a sufficiently low salt concentration (5 mM tris pH 8) to prevent reannealing.

DISCUSSION

We were unable to duplicate the data of Morgan and Wells showing triplex formation between dTC:dGA and poly rUC (8). Also, no triplex formation could be demonstrated between either dTTC:dGAA and rUUC or dTCC:dGGA and rUCC. The problem apparently exists with the RNA's isolated, which appear to contain secondary structure.

There are four possibilities to be considered in attempting to define the secondary structure present in the polypyrimidine RNA preparations. Using poly rUC as an example, the following four structures may be considered:

1) A triplex having the composition dTC:dGA:rU$\overset{+}{\mathrm{C}}$. This is unlikely because the polynucleotide isolation by column chromatography was carried out using a buffer of low ionic strength (10 mM tris pH 8, 0.1 mM EDTA). Likely such a triplex would not form under these pH and ionic strength conditions.
2) A triplex having the composition $(\mathrm{rUC})_2$:dGA. Again this is probably unlikely because of the buffer conditions to which the preparations were subjected.
3) A duplex, of composition dGA:rUC. If this duplex is to be considered a possibility, then it must be concluded that DNase I acts on the dGA strand to a limited extent, if at all. The preference of DNase I for pyrimidine-containing DNA's would support this premise (18). The test for DNA contamination of the RNA preparations indicated that little or no poly rUC was synthesized using the template(s) present. If the poly rUC preparation had the composition dGA:rUC, then it also must be

postulated that this polymer serves as a poor template for poly rUC synthesis.

4) Poly rUC having secondary structure. This possibility seems unlikely, because polypyrimidines characteristically are unstructured.

The net fold synthesis attainable for the polypyrimidine RNA's ranged from 5 fold for a new enzyme preparation to 1 - 2 fold for an older preparation. Polypyrimidine RNA synthesis plateaued after approximately one hour, and no further RNA synthesis was observed. This plateauing of polypyrimidine RNA synthesis was not observed previously by Morgan (12).

All of these results are consistent with the formation of a DNA-RNA duplex and single-stranded polypyrimidine DNA during the transcription process. It would be necessary to postulate that the DNA-RNA duplex serves as a very poor template for further synthesis of RNA which is complementary to the DNA. Also, the DNA strand of the DNA-RNA duplex must be DNase I resistant under the conditions used.

It has been shown by X-ray crystallography that the sodium salt of a DNA-RNA duplex is in the A configuration (19). This is to say that the helix has a pitch of 28 Å with 11 nucleotide pairs per helix pitch. The base pair tilt is 10 - 20°. The sodium salt of DNA in the B configuration has a pitch of 34 Å with 10 nucleotide pairs per helix pitch, and the base pairs are not tilted. Thus the configuration of the DNA-RNA duplex may be sufficiently different from the DNA duplex to inhibit DNase I degradation of the DNA strand.

The preference of DNase I for pyrimidine-containing substrates may enhance the resistance of a DNA-polypyrimidine RNA duplex to

DNase I degradation.

In a 'normal' transcriptive process, the DNA likely undergoes local strand separation, but complete displacement of a DNA strand by the RNA product doesn't occur. Strand displacement during an RNA polymerase reaction has been studied by Chamberlain (20). He analyzed the components present after dI:dC directed poly rC and poly rI synthesis. Quantitation of the components was carried out either by melting or density gradient analysis. The results are shown below:

	COMPONENTS ANALYZED	MμMOLES ADDED	MμMOLES RECOVERED
dI:dC directed rC synthesis	dI:dC	39	35
	dI:rC	0	4
dI:dC directed rI synthesis	dI:dC	43	7
	rI:dC	0	36

The order of helical stabilities, as determined from T_m values is:

$$dI{:}rC < dI{:}dC < rI{:}dC < rI{:}rC$$

In dI:dC directed poly rC synthesis almost all the template is recovered as dI:dC, which has greater helical stability than dI:rC. However, in dI:dC directed poly rI synthesis, most of the template is recovered as rI:dC, which shows greater helical stability than the input template dI:dC.

No simple generalization exists from which the relative order of stabilities in a homopolymer pair series can be predicted. The order of helical stabilities of A:T and G:C base pairs is:

$$dA{:}rU < rA{:}rU < rA{:}dT < dA{:}dT$$

$$\text{and } dG{:}dC < dG{:}rC < rG{:}dC < rG{:}rC \quad (21).$$

With reference to our results, if the order of helical stabilities is dTC:dGA < rUC:dGA, then the isolation of a DNA-RNA duplex is not too surprising.

Since this strand displacement during transcription of dTC:dGA was not observed previously (8), it is proposed that RNA polymerase may exert an effect on RNA strand displacement and DNA rewinding.

Numerous protein factors have been isolated which modify the activity of core RNA polymerase (22). Core polymerase has the subunit composition $\alpha_2\beta\beta'$. A subunit sigma (σ) provides the core polymerase with specificity for promoter recognition. A psi (ψ) factor has been isolated which appears to regulate the initiation of a class of transcriptional units. The function of a small polypeptide chain, ω, found associated with the enzyme, is unknown. The existence of numerous control elements for RNA polymerase has been shown. Further study into the mechanism and control of RNA polymerase activity may reveal a mechanism which exerts control over product strand displacement and template rewinding. From our studies it would appear that the control element involved is storage labile under our conditions.

The problem at this point in our studies became one of investigating the mechanism of RNA polymerase action. Since the main aim of our work was to investigate the properties of triplexes, our efforts were now channeled into characterizing the triplex dA:dT:rU, the components of which were more readily available.

CHAPTER 2

CHARACTERIZATION OF THE TRIPLEX dA:dT:rU

INTRODUCTION

The triplex dA:dT:rU has been characterized by Riley et al. with respect to density gradient centrifugation and thermal melting (21). They showed that cesium sulfate density gradient centrifugation of a sample containing dA:dT and poly rU in a phosphate molar ratio of 2:1 yielded a single band. This indicated that the complex contained both dT and rU paired in the same molecule and that a mixture of dA:$(dT)_2$ and dA:$(rU)_2$ had not been formed. They determined a cesium sulfate buoyant density at 25^o of 1.432 g/cc for dA:dT, 1.650 g/cc for poly rU, and 1.536 g/cc for dA:dT:rU.

The results of their heat denaturation studies indicated that under the wide range of sodium ion concentrations tested, the poly rU strand was melted off first, followed by melting of the DNA.

The following studies were undertaken to investigate the properties of the triplex dA:dT:rU, with the eventual aim of isolating triplexes from biological systems.

MATERIALS AND METHODS

^{3}H-labeled deoxyribonucleoside triphosphates and ^{14}C-labeled polyuridylate, specific activity 0.31 Ci/mole, were purchased from Schwarz BioResearch, Inc. Aquasol and spermine-^{14}C tetrahydrochloride [bis-(aminopropyl)-tetramethylene-1,4-^{14}C-diamine·4 HCl] with a specific activity of 12.9 Ci/mole were purchased from New England Nuclear. Unlabeled spermine tetrahydrochloride was obtained from Calbiochem.

Pancreatic ribonuclease (specific activity 3900 units/mg) was obtained from Worthington Biochemical Corporation. Micrococcus luteus DNA polymerase was purchased from Miles Laboratories, Inc. It was supplied as a frozen solution in phosphate buffer, with a specific activity of over 200 units/mg protein.

Absorption spectra and molar extinction coefficient determinations were carried out as described in Chapter 1.

Mixing curves were obtained by the method of continuous variations (23). Known amounts of one polymer were mixed with a known amount of another polymer in a quartz cuvette, and the optical density was monitored at an appropriate wavelength.

Band sedimentation was done in a Spinco Model E ultracentrifuge using a Vinograd cell. 20 μl of DNA or RNA at a concentration of approximately 2 OD/ml in buffer containing 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$ was layered at low speed onto buffer containing 1.0 M NaCl, 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$. Centrifugations were done at 56,000 rpm and 20°.

pH melting profiles were determined by adding successive aliquots of either KOH or HCl to a cuvette containing the appropriate polymer.

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

The pH and optical density at 260 nm were measured after each addition. pH measurements were made using a Beckman Expandomatic SS-2 pH meter with a Beckman glass electrode and ceramic junction. The pH values above 11 were corrected for sodium ion concentration. The manufacturer's correction for this glass electrode in 0.5 M NaCl at 25° is 0.10 pH units at pH 11 and 0.53 pH units at pH 12.5. Finally, the weight of the cuvette and polymer solution was recorded after each acid or alkali addition, in order to determine the solution volume accurately.

Preparation of E.Coli DNA polymerase fraction 7 was described in Chapter 1. Synthesis of RNA was monitored as described in Chapter 1. When duplex DNA was supplied as the template, 36 mμmoles/ml dA:dT was provided. However, when triplex was supplied as the template, 54 mμmoles/ml dA:dT:rU was added.

Synthesis of DNA was followed by the incorporation of radioactive deoxyribonucleoside triphosphates into acid insoluble polynucleotide at 37°. A standard E.Coli or M.luteus DNA polymerase assay contained in 0.10 ml:

50 mM tris pH 8
15 mM $MgCl_2$
2 mM ^{3}H-dATP (specific activity 5 Ci/mole)
2 mM TTP
54.6 mμmoles/ml dA:dT
40 μg/ml E.Coli DNA polymerase fraction 7
or 40 units/ml M.luteus DNA polymerase

When assaying the triplex dA:dT:rU for its replication potential, an additional 27.3 mμmoles/ml poly rU was added. To quantitate the radioactive deoxyribonucleoside triphosphate incorporated, the filter paper assay, as described previously, was used.

Large quantities of dA:dT were synthesized from dATP and TTP by

incubating E.Coli DNA polymerase with rA:rU at 45°. A typical reaction mixture contained:

50 mM tris pH 8
15 mM $MgCl_2$
4 mM dATP
4 mM TTP
74.8 mμmoles/ml rA:rU
39 μg/ml E.Coli DNA polymerase fraction 7

Synthesis of DNA was followed by measuring ethidium bromide fluorescence, as described previously. An unwanted side reaction, dAT:dAT synthesis, was monitored by measuring fluorescence remaining after heating to 100° for 2 minutes and quick cooling a sample of reaction mixture diluted 400 - 1000 fold with buffer containing 2 mM tris pH 8.5, 0.2 mM EDTA, 0.5 μg/ml ethidium bromide (24). After 9 hours synthesis approximately 25 'net fold' synthesis of the rA:rU was obtained. The reaction mixture became very viscous as the reaction proceeded. If synthesis was allowed to continue for 12 hours, a solid gel was formed. Normally the dAT:dAT contamination was less than 3%. After a 2 fold dilution with 50 mM tris pH 8, the dA:dT was purified by passage over a Biogel A-0.5m (50-100 mesh) column, from which the DNA appeared in the void volume.

^{14}C-labeled dA:dT was prepared by the procedure described above, except that ^{14}C-dATP and/or ^{14}C-TTP were supplied. They were diluted 100 fold with unlabeled dATP or TTP, to a final specific activity of about 0.4 Ci/mole before being added to the reaction mixture.

DNase I activity was followed by the loss of ^{14}C-label from acid insoluble polynucleotide. A typical reaction mixture contained in 0.10 ml:

50 mM Na phosphate pH 7.3
10 mM $MgCl_2$
5 μg/ml DNase I
90 mμmoles/ml ^{14}C-labeled DNA

When a triplex was provided as substrate, an additional 45 mμmoles/ml RNA was added. The reaction mixture was incubated at 37° and aliquots were removed at appropriate times. The filter paper assay described previously was used to quantitate DNase I degradation of polynucleotides.

Pancreatic ribonuclease activity was assayed by the same method as that described for DNase I. An RNase assay mixture contained:

50 mM Na phosphate pH 7.3
10 mM $MgCl_2$
25 μg/ml pancreatic RNase
55 mμmoles/ml ^{14}C-labeled RNA

When a triplex was provided as substrate, an additional 110 mμmoles/ml DNA was added to the reaction mixture.

Equilibrium dialysis was carried out in Lucite cells which were made similar to those of Englund et al. (25). The only adaptation of their design was an increase in the chamber volume from 30 μl to 50 μl. The membrane was dialysis tubing which had been boiled in three changes of 5% Na bicarbonate, 10 mM tris pH 8, 0.1 mM EDTA, for 5 minutes each time, and stored in the same buffer solution. Before use, the membrane was rinsed with distilled water and wiped dry. The cell was assembled by placing a 1 cm^2 piece of membrane between the two chambers in each set, and then clamping the whole cell together tightly in a metal holder. The chambers were loaded with an Agla micrometer syringe fitted with a 1 ml syringe barrel and metal needle #20. 35 μl of DNA (230 mμmoles/ml) was added to one side of the membrane, and 35 μl of the ^{14}C-spermine (26,000 cpm/mμmole) was added to the other side. Each was in a buffer containing 5 mM Na cacodylate

pH 6.8. The cells were sealed with Scotch tape (the tape did not contact the liquid), and their contents were mixed by rocking on an Eberbach horizontal shaker at 270 oscillations/minute at 24°. After equilibrium had been reached 20 μl samples were withdrawn from each chamber and counted in 10 ml of dioxane-based scintillation fluid (Aquesol).

RESULTS

Absorption Spectra and Molar Extinction Coefficient Determinations of dA:dT and poly rU

The absorption spectra of dA:dT and poly rU in 1 M NaCl, 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$ are shown in figure 8. The DNA showed a λ_{max} at 257 nm, and a 260/280 absorbance ratio of 1.75. The RNA showed a λ_{max} at 260 nm, and a 260/280 absorbance ratio of 2.96.

Duplicate molar extinction coefficient determinations of dA:dT were carried out using both micrococcal nuclease plus spleen phosphodiesterase, and DNase I plus snake venom phosphodiesterase. An average of the four determinations gave a value of 6.6×10^3 at 260 nm and 6.7×10^3 at 257 nm (λ_{max}). The two methods of degradation gave values which agreed within 1%. These values are slightly higher than those reported by Riley et al. (22). Also, the λ_{max} is slightly lower than the value reported previously (22).

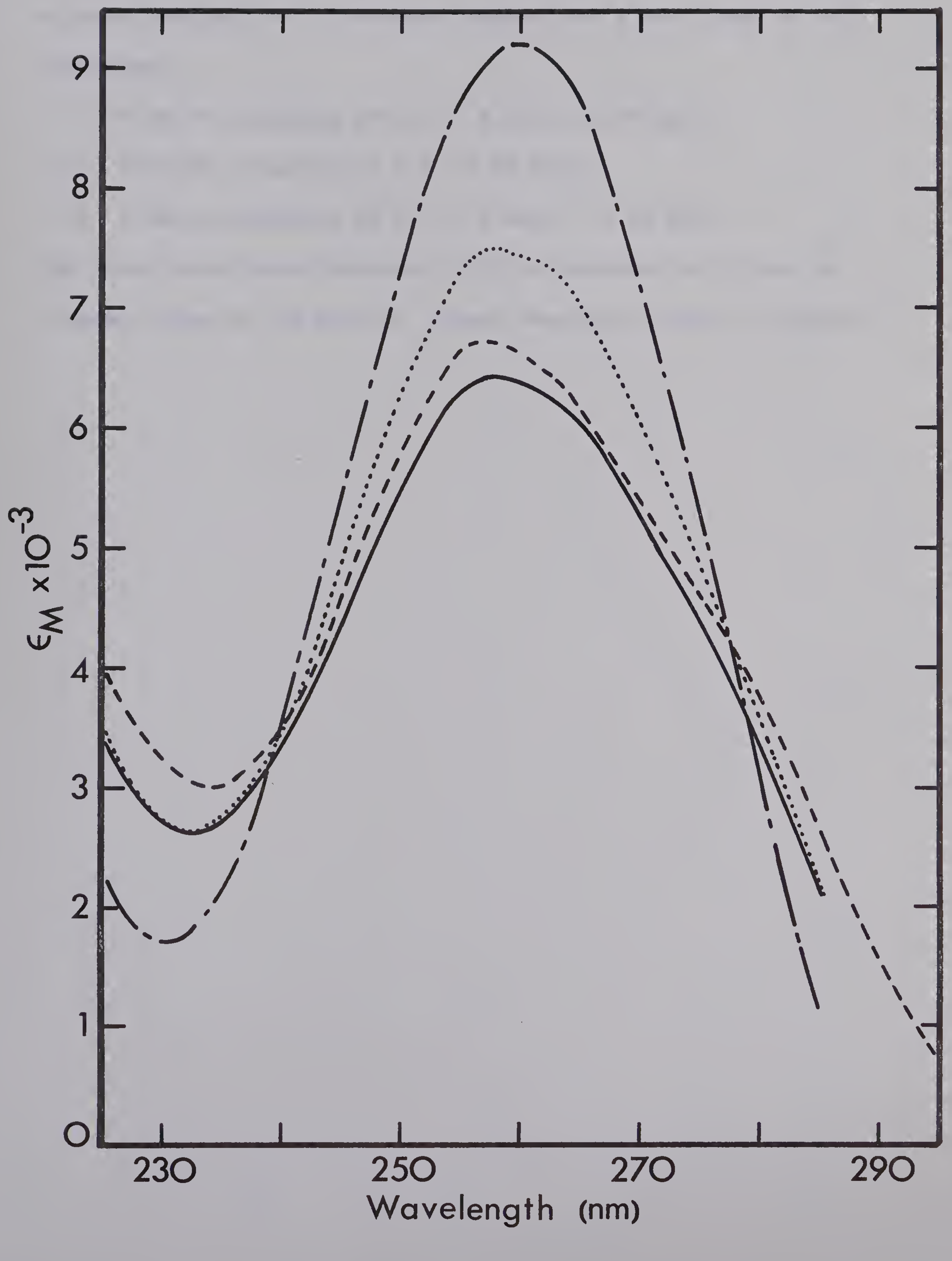
Duplicate molar extinction coefficient determinations of poly rU were carried out using micrococcal nuclease and spleen phosphodiesterase. The averaged value was 9.2×10^3 at 260 nm.

The theoretical and observed absorption spectra of a mixture containing dA:dT and poly rU at phosphate molar ratios of 2:1 are shown in figure 8. The theoretical spectrum was calculated by summing the contributions of dA:dT and poly rU, assuming no hypochromicity results from their combination in solution. Their interaction resulted in a hypochromic effect between 240 nm and 285 nm, the maximum effect being between 258 nm and 264 nm.

Figure 8. Absorption spectra of dA:dT, poly rU, dA:dT:rU (actual), and dA:dT plus poly rU (theoretical). The spectra were determined in 1 M NaCl, 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$.

----dA:dT; —— dA:dT:rU (actual);

-—-poly rU; ······· dA:dT plus poly rU (theoretical)


$\epsilon_M \times 10^{-3}$
0
1
2
3
4
5
6
7
8
9
230
250
270
290
Wavelength (nm)

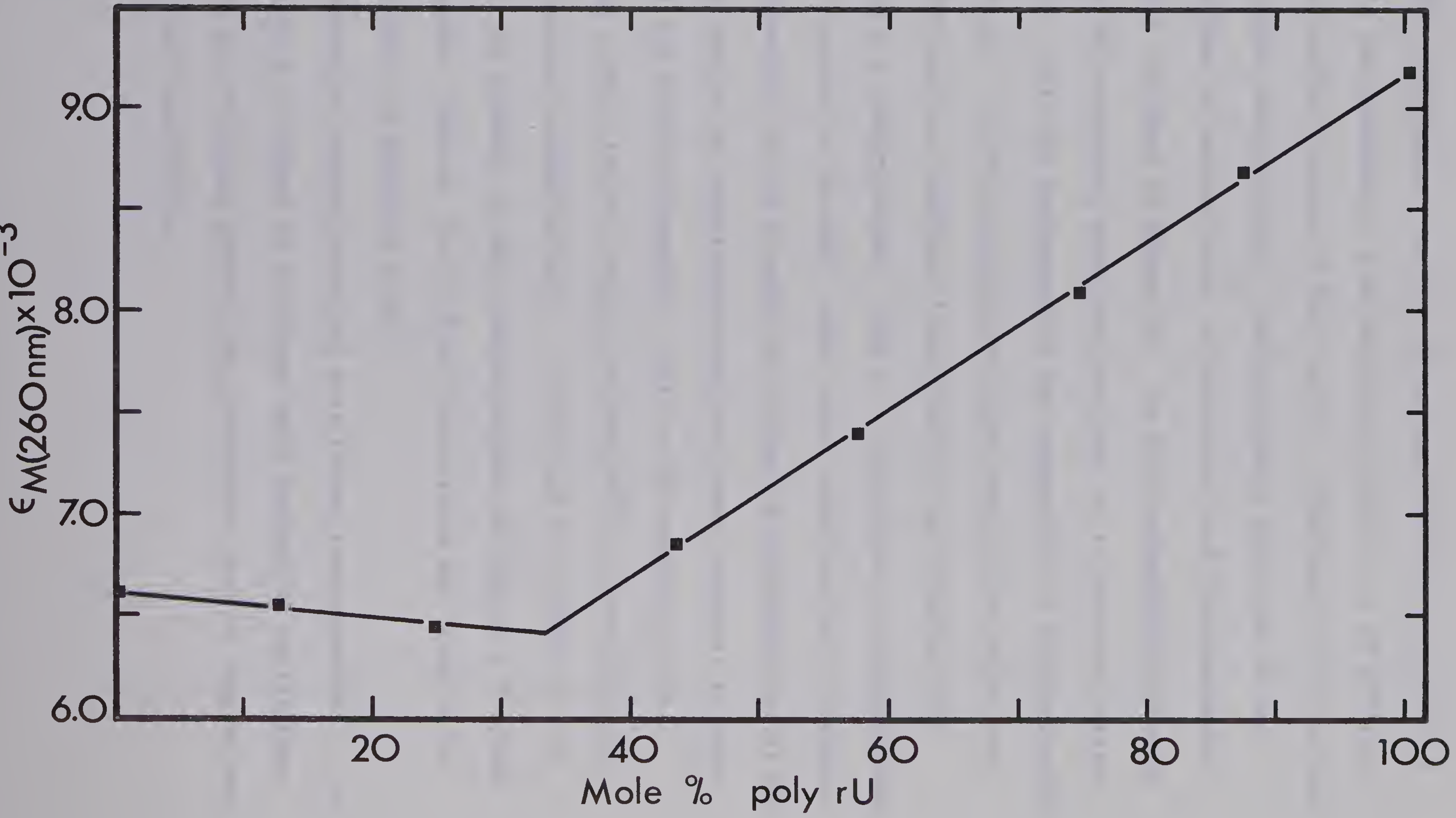
Mixing Curves

A wavelength of 260 nm was chosen for determining mixing curves of dA:dT and poly rU. They were repeated for a wide range of salt conditions:

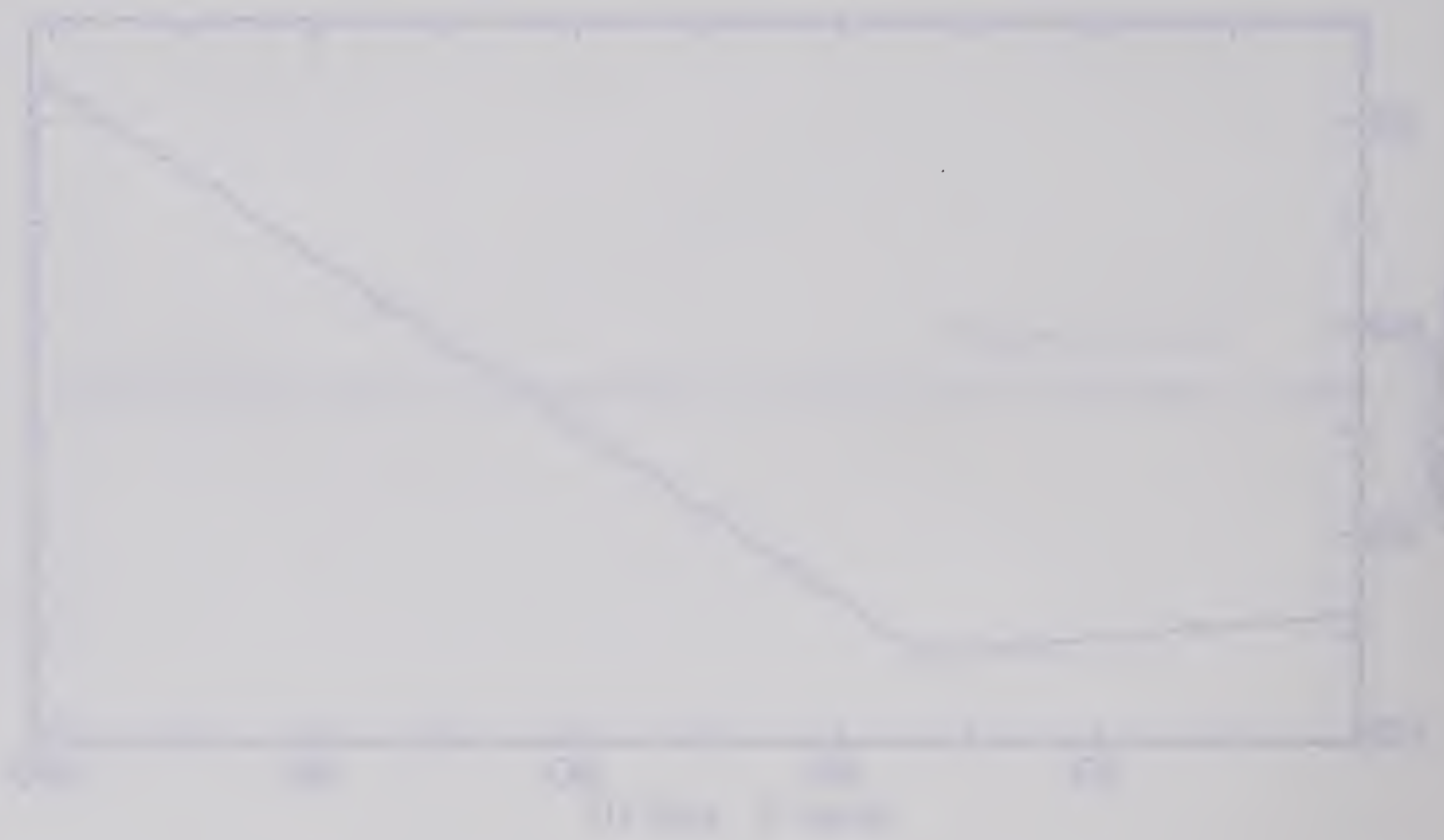
1) 50 mM Na phosphate pH 7.3, 1 M NaCl, 10 mM $MgCl_2$;
2) 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$;
3) 50 mM Na phosphate pH 7.3, 1 M NaCl, 0.1 mM EDTA.

The three curves were identical, with an intersection of the two straight lines at 33% poly rU. These results are shown in figure 9.

Figure 9. Mixing curve for dA:dT and poly rU. Mixing was done in 1 M NaCl, 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$.



$\epsilon_{M(260nm)} \times 10^{-3}$
9.0
8.0
7.0
6.0
20
40
60
80
100
Mole % poly rU



Triplex Formation as a Function of KCl and NaCl Concentration

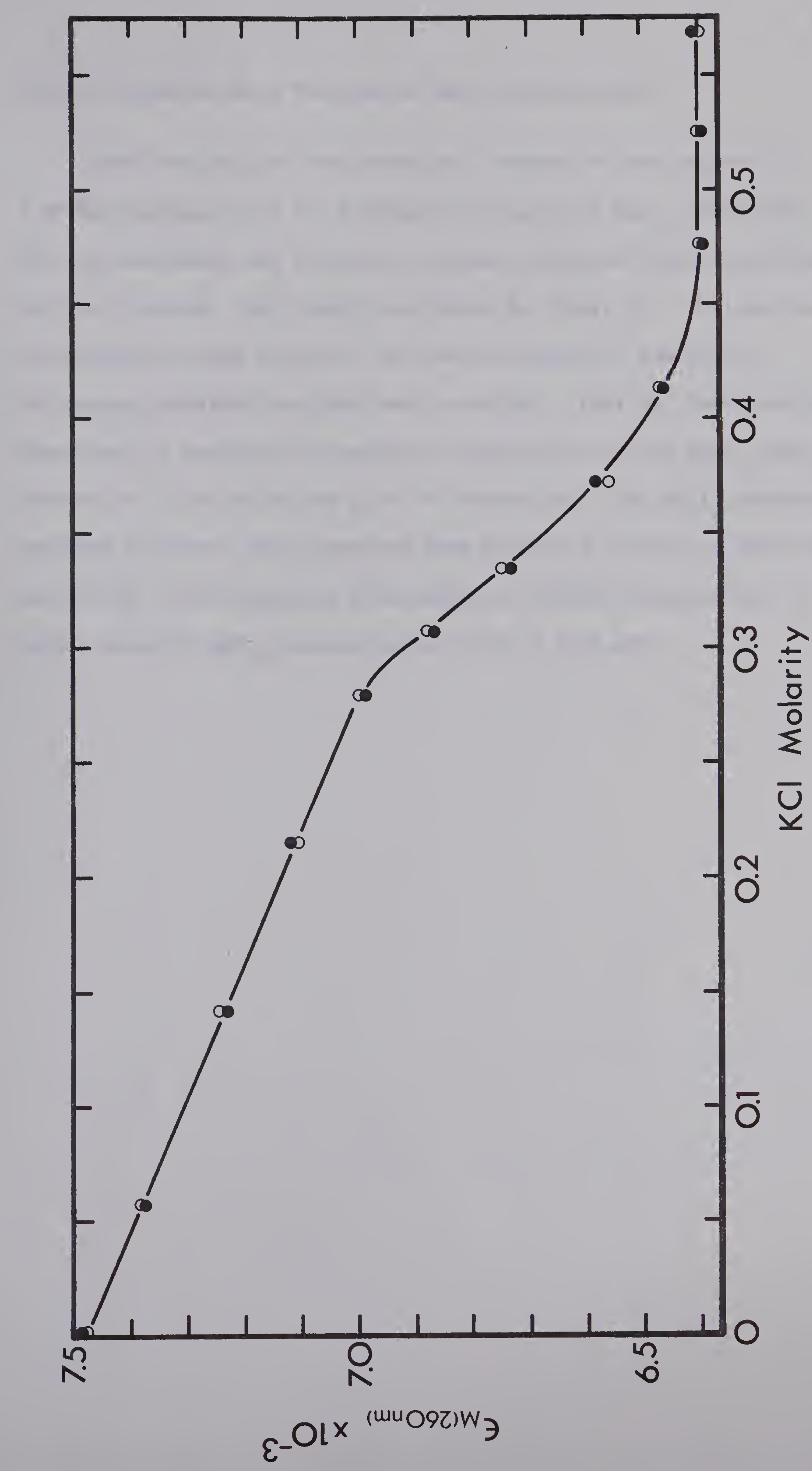
These experiments were done by mixing dA:dT and poly rU in a cuvette in the presence of 5 mM Na phosphate pH 7.3, 1 mM EDTA, and adding successive aliquots of NaCl or KCl. Absorbancy at 260 nm was recorded after each addition. The experiments were done at two concentrations of nucleic acid: 46 mμmoles/ml and 196 mμmoles/ml. The results are shown in figure 10. The KCl concentration at which there was 50% conversion from dA:dT and poly rU to dA:dT:rU triplex was 0.35 M. This KCl concentration was independent of polynucleotide concentration. At KCl concentrations less than 0.28 M, where the DNA and RNA were not combined, hypochromicity was observed with increasing KCl concentration. This is a reflection of conformational changes occurring in the DNA. Under these conditions poly rU showed no hypochromicity in the presence of increasing concentrations of KCl.

The results of experiments using NaCl were identical to those using KCl for dA:dT:rU formation. This is in contrast to the conditions under which the triplex dTC:dGA:rU$\overset{+}{\text{C}}$ is formed (Dr. A.R. Morgan, personal communication). In this case the triplex is not formed in the presence of NaCl concentrations as high as 1 M unless Mg^{+2} is added. However, in 0.2 M KCl the triplex was found to be formed without the addition of Mg^{+2}.

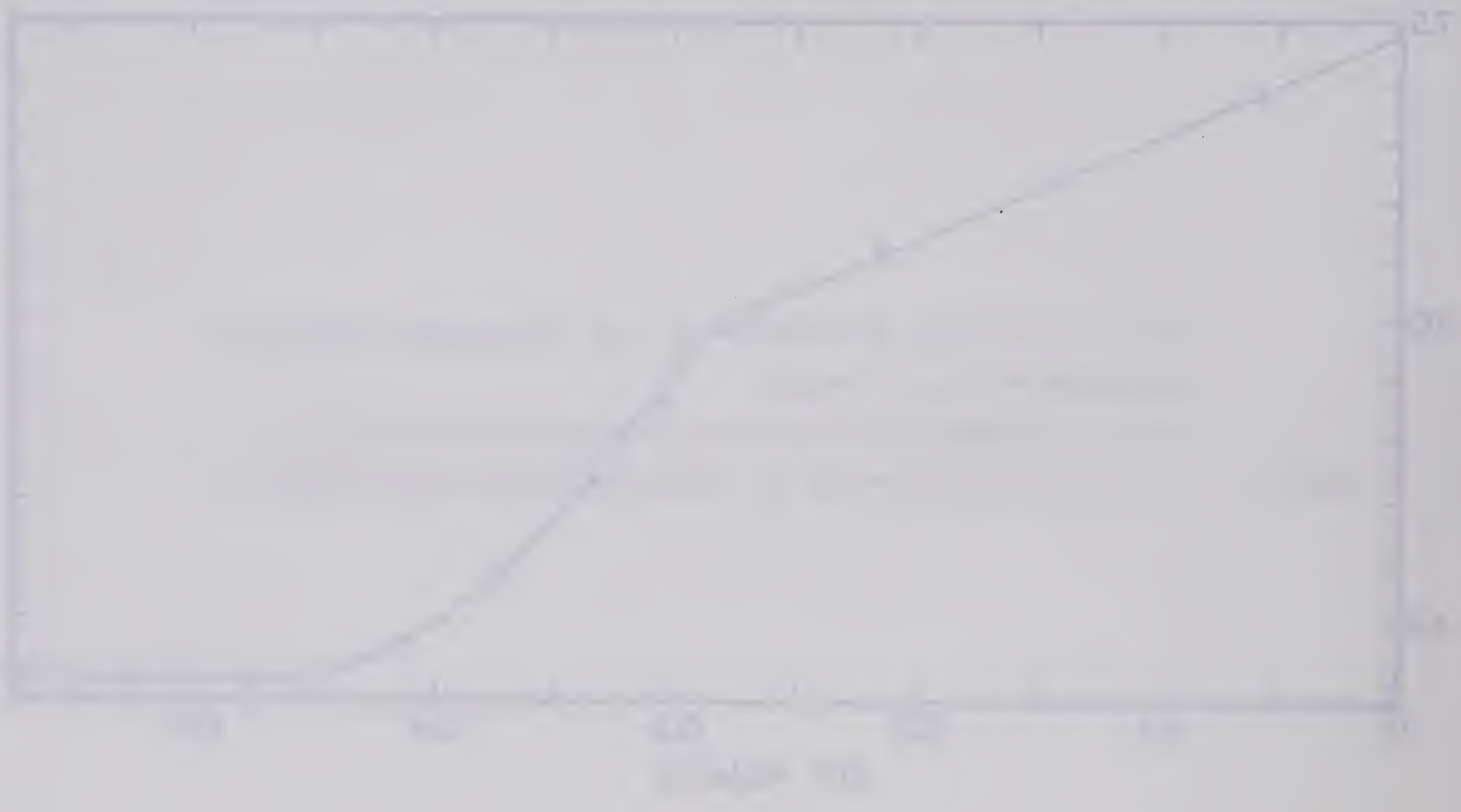
Interaction between dA:dT and poly rU was time dependent. The values plotted in figure 10 are those which existed at equilibrium. As the DNA and RNA began interacting, absorbancy took as long as two hours to reach equilibrium.

Figure 10. KCl titration of dA:dT plus poly rU. Successive aliquots of KCl were added to a solution containing dA:dT and poly rU at a phosphate molar ratio of 2:1 in 5 mM Na phosphate pH 7.3, 1 mM EDTA.

○——○ 46 mμmoles/ml nucleotide P_i; ●——● 196 mμmoles/ml nucleotide P_i



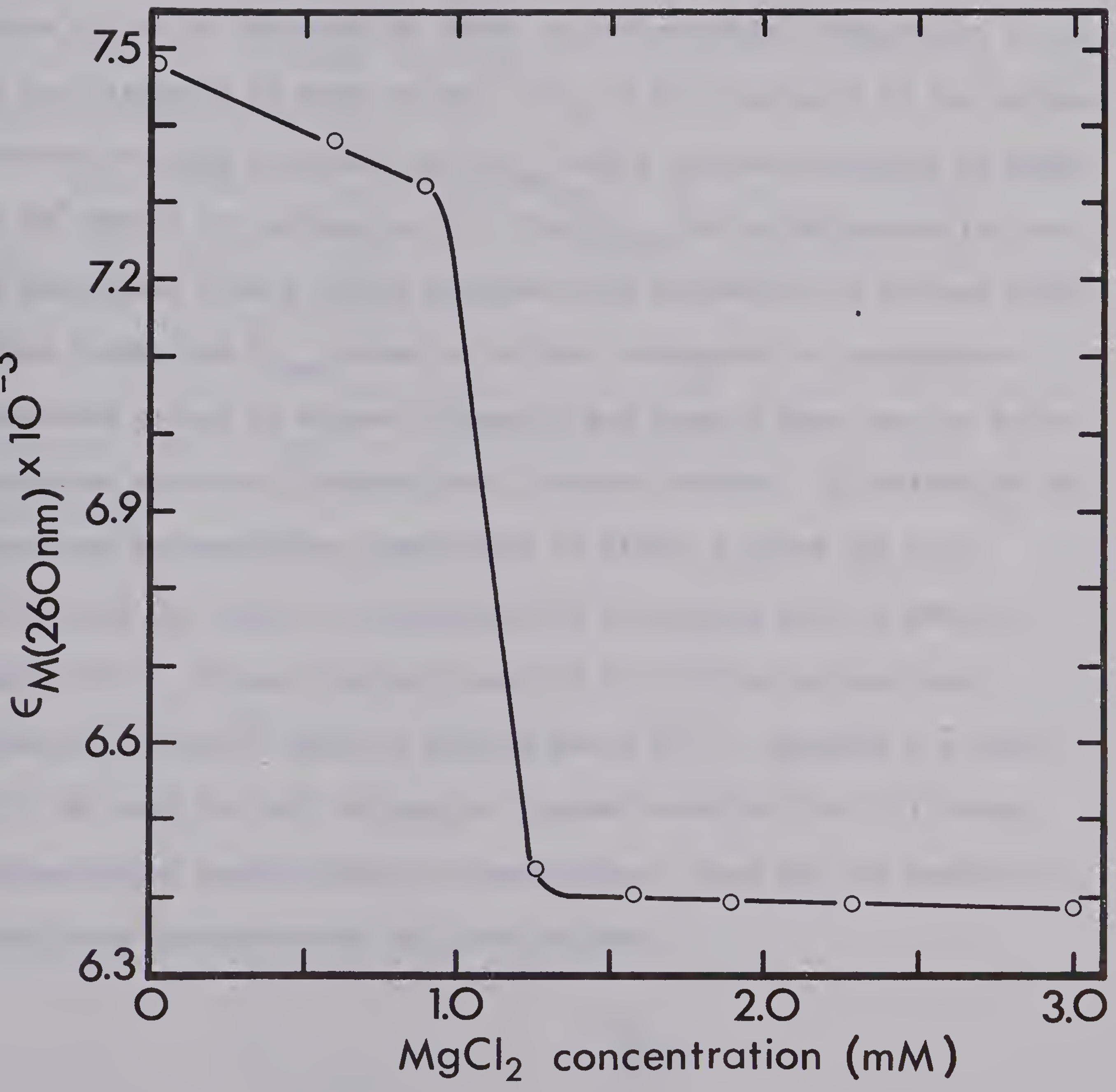
7.5
7.0
6.5
ε_M(260nm) x10^-3
0
0.1
0.2
0.3
0.4
0.5
KCl Molarity



Triplex Formation as a Function of $MgCl_2$ Concentration

dA:dT and poly rU were mixed in a cuvette in the presence of 5 mM Na phosphate pH 7.3. Successive aliquots of $MgCl_2$ were added, and the absorbance was recorded after each addition, when equilibrium had been reached. The results are shown in figure 11. Two levels of polynucleotide were studied: 48 mμmoles/ml and 203 mμmoles/ml. The results obtained for each were identical; that is, there was no dependence of nucleotide phosphate concentration on the $MgCl_2$ level required to allow dA:dT and poly rU interaction. The $MgCl_2$ concentration required to permit 50% conversion from dA:dT and poly rU to dA:dT:rU was 1.1 mM. The conversion from duplex to triplex occurred over a narrow range of $MgCl_2$ concentrations (0.90 - 1.25 mM).

Figure 11. $MgCl_2$ titration of dA:dT plus poly rU. Successive aliquots of $MgCl_2$ were added to a solution containing dA:dT and poly rU at a phosphate molar ratio of 2:1 in 5 mM Na phosphate pH 7.3.


$\epsilon_{M(260nm)} \times 10^{-3}$
7.5
7.2
6.9
6.6
6.3
0
1.0
2.0
3.0
$MgCl_2$ concentration (mM)

Band Velocity Sedimentation of dA:dT, poly rU, and dA:dT:rU

The sedimentation coefficients of dA:dT, poly rU, and dA:dT:rU were corrected to $S_{20,w}$ by the equation:

$$S_{20,w} = S\left\{\frac{\eta_t}{\eta_{20}}\right\}\left\{\frac{\eta}{\eta_o}\right\}\left\{\frac{1-\bar{v}\rho_{20,w}}{1-\bar{v}\rho}\right\},$$

where η_t is the viscosity of water at the operating temperature t, η_{20} is the viscosity of water at 20^o, η/η_o is the viscosity of the solvent relative to that of water, and $\rho_{20,w}$ and ρ are the densities of water at 20^o and of the solvent at t^o. The $S^o_{20,w}$ for a polynucleotide can be determined from a single Vinograd band sedimentation because under these conditions $S_{20,w}$ shows no polymer concentration dependence. Published values of solvent viscosity and density were used to solve the above equation (International Critical Tables). To determine the intrinsic sedimentation coefficient of dA:dT, a value for $\bar{v}$ of 0.556 cc/g was used, as determined for the sodium salt of DNA by Hearst (26). Values ranging from 0.48 to 0.578 cc/g have been reported for the $\bar{v}$ value of various RNA's (27). Assuming a $\bar{v}$ value of 0.556 cc/g for poly rU and the triplex dA:dT:rU, the following sedimentation coefficients were determined. They are the averages of triplicate determinations for each polymer.

	$S^o_{20,w}$
dA:dT	21.2s
poly rU	3.5s
dA:dT:rU	30.4s

The sedimentation coefficient of the triplex is 44% greater than that of the DNA. $S^{o}_{20,w}$ determinations of various dA:dT preparations yielded results ranging from 17s to 26s. However, the triplex in each case had a sedimentation coefficient 43 - 47% greater than that of the DNA.

The stoichiometry of interaction between dA:dT and poly rU was confirmed by the presence of a single band in the sedimentation profiles when the sample contained a phosphate molar ratio of 2 dA:dT : 1 poly rU. If the sample contained a higher molar ratio of DNA : RNA, then two sedimenting bands were observed.

Using Studier's equation for native DNA, $S^{o}_{20,w} = 0.0882\ M^{0.346}$ (14), the molecular weight of dA:dT having a sedimentation coefficient of 21.2s would be 7.8 X 10^{6}.

No increase in sedimentation coefficient of dA:dT was observed when poly rAU was added at a phosphate molar ratio of 2 dA:dT : 1 poly rAU.

pH Melting of dA:dT, poly rU, and dA:dT:rU

pH melting experiments were done at 25° in the presence of either 0.5 M NaCl or 0.5 M KCl. It had been shown previously that at these salt concentrations a triplex would form. $MgCl_2$ could not be added because at alkaline pH an insoluble precipitate $Mg(OH)_2$ was formed. Titration with acid or alkali was done from pH 7.3. To obtain a melting curve of dA:dT:rU, the DNA and RNA were first mixed in a phosphate molar ratio of 2:1.

The melting curves obtained in the presence of KCl and NaCl were identical. The results of acid and base titration of the 3 polymers in 0.5 M KCl are shown in figure 12. Each curve represents an average of 3 determinations.

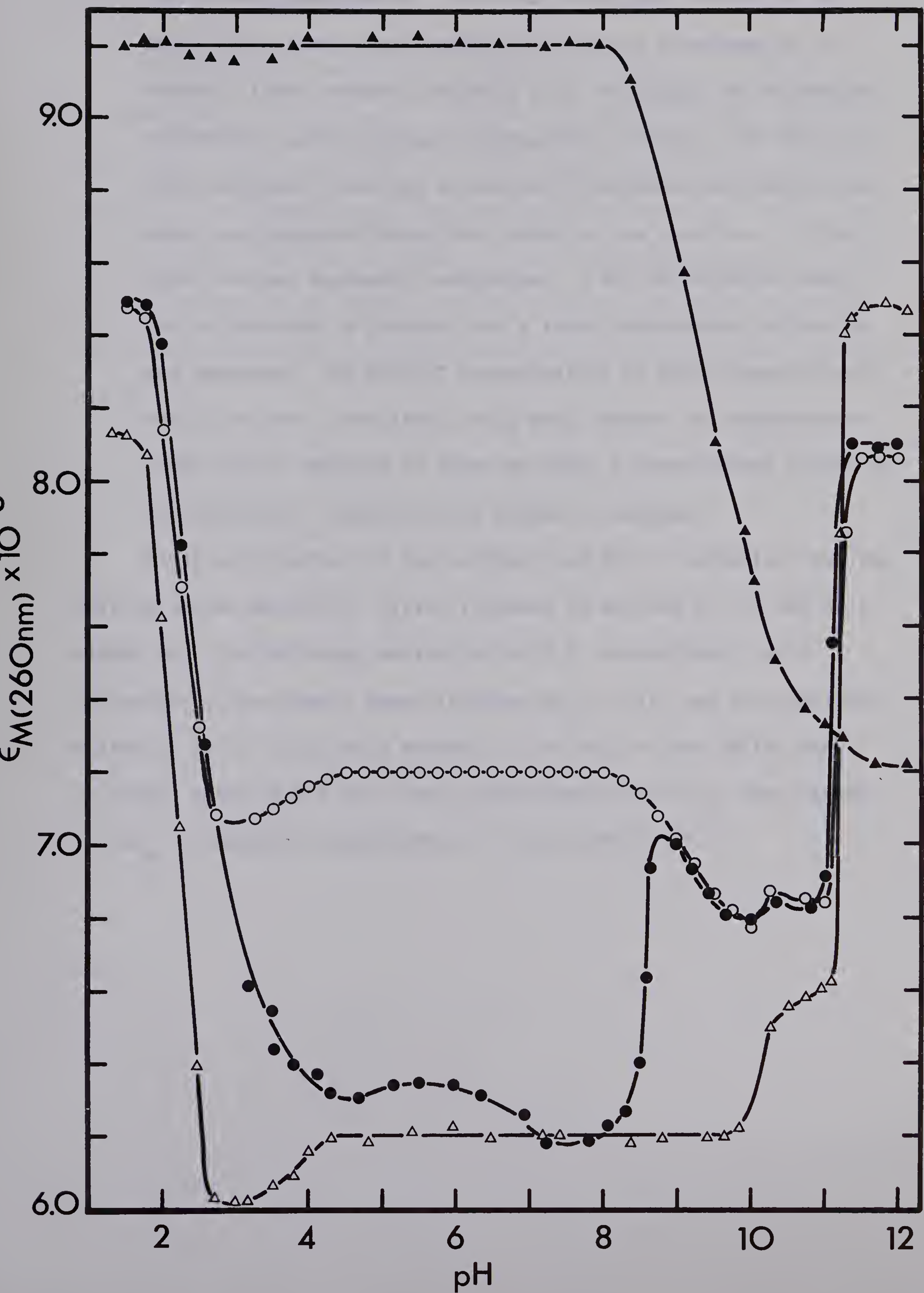
Two points are noteworthy for the melting profile of dA:dT.

1) Acid titration showed a decrease in absorbancy between pH 4.2 and 3.3. When studying acid titration of poly dA, Bollum et al. noted this same hypochromicity, which they attributed to the formation of a poly dA acid helical structure (27). Riley et al. refuted this interpretation, and suggested that it was due to aggregation of the polymer (21). To resolve this question, the acid titration from pH 6 to pH 2.5 was followed at 320 nm and 660 nm, as well as at 260 nm. No increase in turbidity could be detected at either of the two additional wavelengths studied. Hence our data would argue against aggregation of poly dA at acid pH.
2) Alkaline titration of dA:dT showed a hyperchromic shoulder between pH 9.8 and 11. This phenomenon was reproducible in two different

Figure 12. pH titration curves for dA:dT, poly rU, dA:dT:rU (actual), and dA:dT plus poly rU (theoretical). Titrations were done from pH 7.3, at 25°. The polymers were in 0.5 M KCl, and either KOH or HCl was added in successive aliquots.

△——△ dA:dT; ●——● dA:dT:rU (actual);

▲——▲ poly rU; ○——○ dA:dT plus poly rU (theoretical)


9.0
8.0
7.0
6.0
ε_M(260nm) x10^-3
2
4
6
8
10
12
pH

DNA preparations tested. rA:rU may have been present in the dA:dT preparations, the contamination being a maximum of 4%. However, later studies indicated that the E.Coli DNA polymerase preparation used contained ribonuclease activity, and thus the rA:rU initially supplied in the dA:dT synthesizing reaction may have been degraded during the course of the reaction. If the rA:rU was not degraded, nonetheless, a 4% contamination would not be expected to produce such a large hyperchromic effect as was observed. The dAT:dAT contamination of dA:dT preparations was 3% or less. Similarly, this small amount of contamination would not be expected to cause as great a hyperchromic effect as was observed. Otherwise, its origin is unknown.

Alkaline titration of the triplex from pH 7.3 indicated that the poly rU strand melted off first, followed by melting of the DNA at a higher pH. The RNA began melting at pH 8.2, concomitantly with ionization of the uracil bases (uridine $pK_a = 9.2$), and was completely melted by pH 9. This early melting of the poly rU was unlike that of dA:dT, which didn't melt until approximately pH 11.2, even though the pK_a of thymidine monophosphate is approximately 9.2.

Effect of SDS on dA:dT:rU

dA:dT:rU was formed in the presence of 1 M NaCl, 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$. Aliquots of sodium dodecyl sulfate were added, and the absorbance at 260 nm was recorded after each addition. The highest final SDS concentration tested was 0.01%. The results showed that the absorbance of the triplex was unaffected under all SDS concentrations studied. Also, under these salt conditions, and in the presence of 0.01% SDS, the DNA and RNA interacted to form a triplex.

Transcription of dA:dT and dA:dT:rU by E.Coli RNA Polymerase

The concentration of RNA polymerase used for these experiments was 80 μg/ml. SDS polyacrylamide gel electrophoresis of this polymerase preparation indicated that it contained approximately stoichiometric amounts of the sigma subunit. The results are shown in figure 13. In the absence of poly rU synthesis, early poly rA synthesis was inhibited approximately 65% by providing the triplex dA:dT:rU instead of dA:dT as template. At times greater than 20 minutes, the observed inhibition was less than 65%. Perhaps this is a reflection of the newly synthesized poly rA complexing with the poly rU of the triplex, thus making more double stranded template available for transcription.

When poly rU synthesis was followed in the absence of poly rA synthesis, an 80% inhibition was observed up to 40 minutes, as shown in figure 14. These results are consistent with the data of Morgan and Wells (8).

That there was no RNase activity present in the RNA polymerase preparation was confirmed by incubating RNA polymerase with ^{14}C-poly rU under the transcription assay conditions, and checking for loss of acid insoluble label with time. Similarly, using ^{14}C-dA*:dT*, the absence of DNase activity in the RNA polymerase preparation was confirmed.

When poly rU was added to a reaction mixture containing dAT:dAT as template, at a phosphate molar ratio of 2 dAT:dAT : 1 poly rU, there was no inhibition of poly rAU synthesis. Similarly, when poly rAU was added to a reaction mixture containing dA:dT as template, there was no inhibition of either poly rA or poly rU synthesis.

These transcription studies were repeated in the presence of

Figure 13. Transcription of dA:dT and dA:dT:rU - poly rA synthesis. The assay mixtures contained 50 mM tris pH 8, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5 mM ^{14}C-ATP, 36 mμmoles/ml dA:dT or 54 mμmoles/ml dA:dT:rU, 0.43 mg/ml *E.Coli* RNA polymerase. Incubation was at 37°.

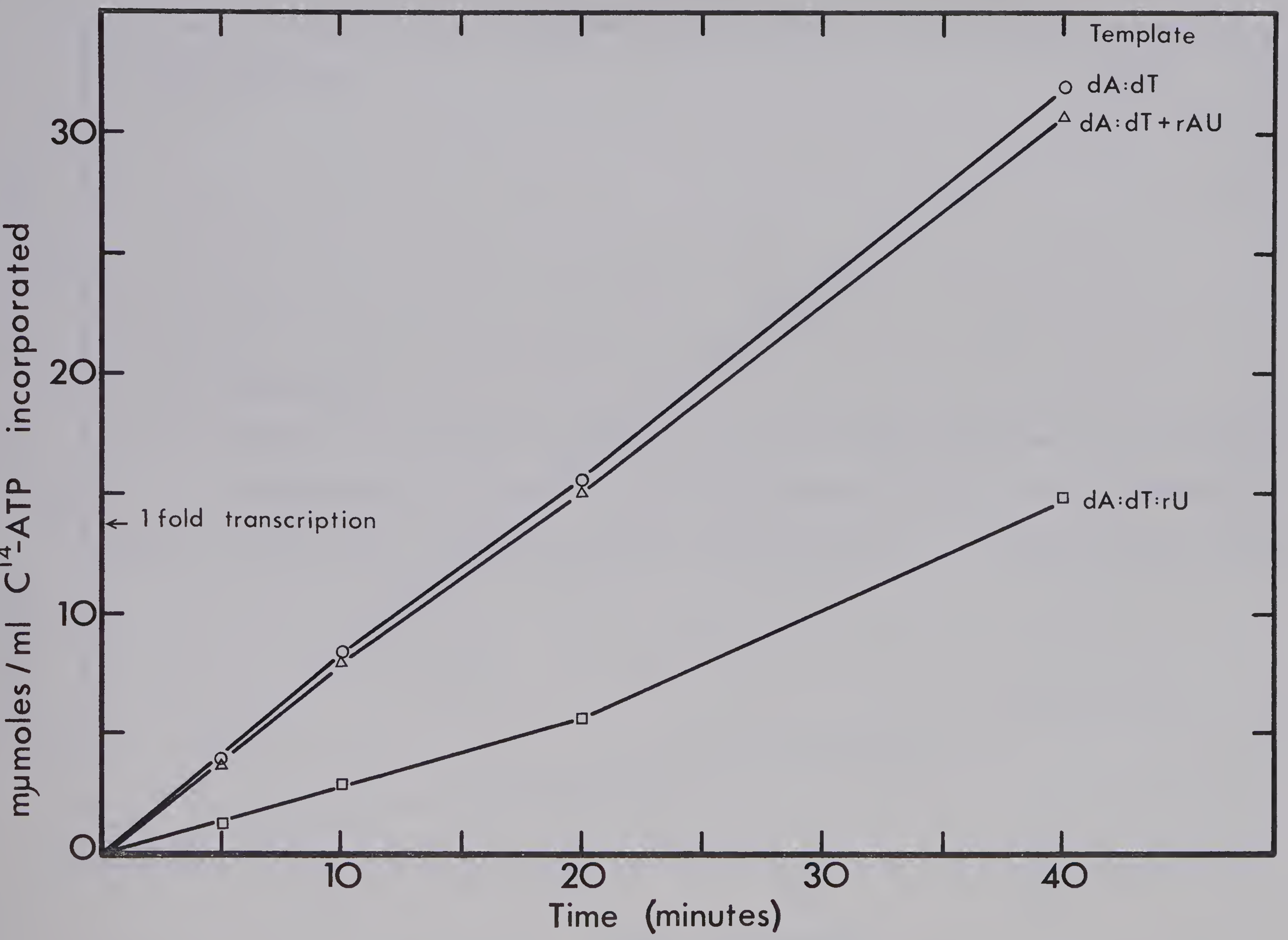
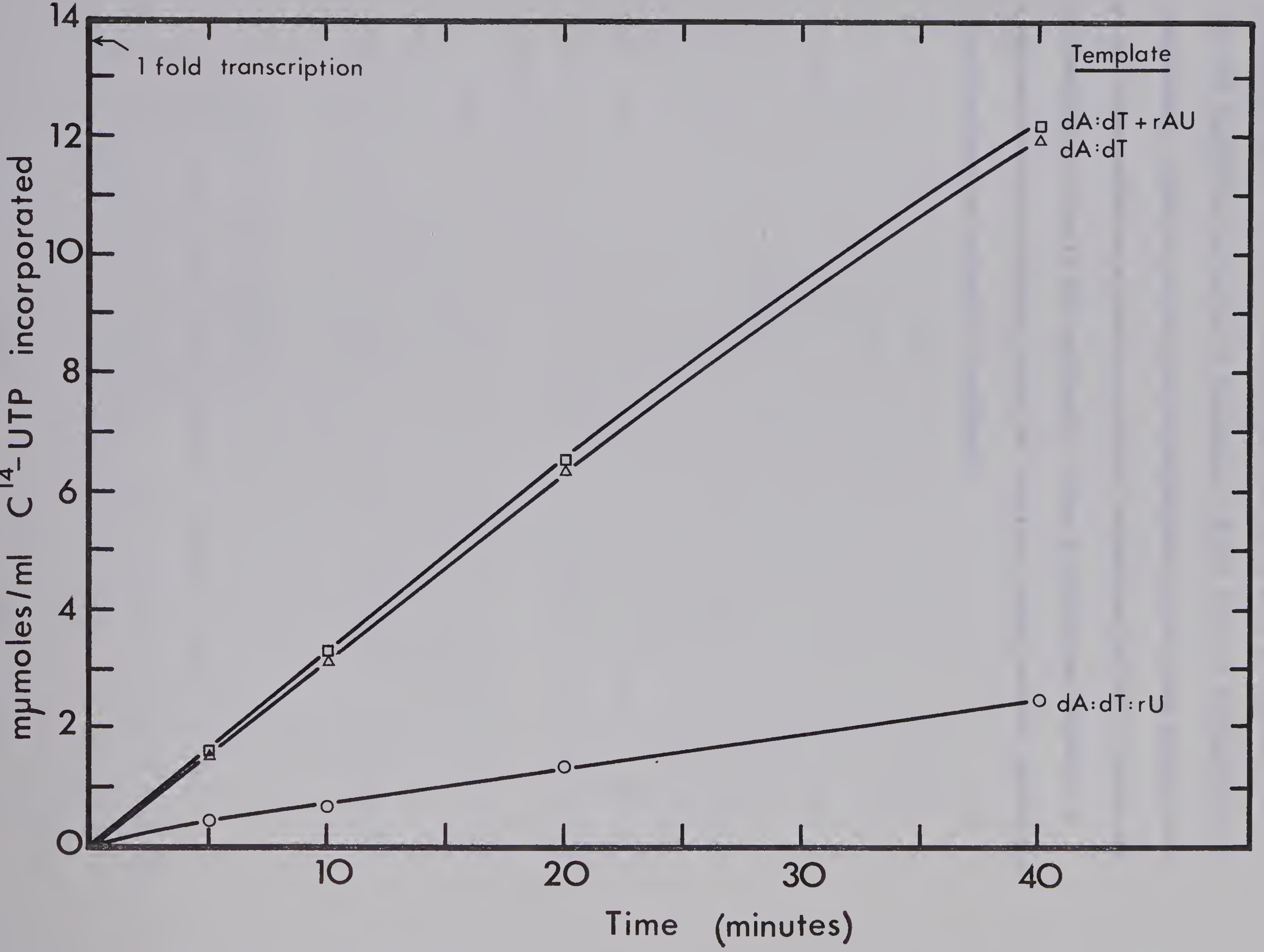

Template
○ dA:dT
△ dA:dT + rAU
□ dA:dT:rU
← 1 fold transcription
mμmoles / ml C^{14}-ATP incorporated
0
10
20
30
10
20
30
40
Time (minutes)

Figure 14. Transcription of dA:dT and dA:dT:rU - poly rU synthesis. The assay mixtures contained 50 mM tris pH 8, 5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.5 mM ^{14}C-UTP, 36 mμmoles/ml dA:dT or 54 mμmoles/ml dA:dT:rU, and 0.43 mg/ml *E.Coli* RNA polymerase. Incubation was at 37°.


1 fold transcription
Template
dA:dT + rAU
dA:dT
dA:dT:rU
mμmoles/ml C14-UTP incorporated
0
2
4
6
8
10
12
14
10
20
30
40
Time (minutes)

0.2 M KCl, the approximate salt concentration thought to exist within an E.Coli cell. When the triplex was provided as a template, there was 30% inhibition of poly rU synthesis, while poly rA synthesis was unaffected. Similar to the control experiments described above, when poly rU was added to a reaction mixture containing dAT:dAT, there was no inhibition of poly rAU synthesis. When poly rAU was added to a reaction mixture containing dA:dT as template, there was no inhibition of either poly rA or poly rU synthesis.

Replication of dA:dT and dA:dT:rU by E.Coli and M.luteus DNA Polymerases

1) E.Coli DNA Polymerase:

The rates of replication of dA:dT and dA:dT:rU by E.Coli DNA polymerase were compared. Because the DNA polymerase preparation used contained some contaminating RNase activity, degradation of the triplex RNA was followed concomitantly with replication of the triplex. These results are shown in figure 15. After 5 minutes of synthesis the rate of replication using dA:dT:rU as a template was about 60% less than the rate using dA:dT as template. However, after 5 minutes of synthesis, already 20% of the poly rU had been degraded from the triplex. Thus in the absence of RNase activity, the inhibition of dA:dT synthesis when a triplex is provided as template may be greater than 60%. The per cent inhibition of dA:dT synthesis decreased with time; this is a reflection of RNase digestion of the poly rU strand in the triplex, as well as production of uncomplexed dA:dT in the DNA synthesizing reaction.

When poly rG was added to an assay mixture containing dA:dT as template, at a phosphate molar ratio of 2 dA:dT : 1 poly rG, no inhibition of dA:dT synthesis was observed. Similarly, poly rU did not inhibit the replication of dAT:dAT.

2) M.luteus DNA Polymerase:

The rates of DNA replication were compared when dA:dT and dA:dT:rU were supplied as templates. Some contaminating RNase activity was shown to exist in the M.luteus preparation used. For this reason, degradation of triplex RNA was followed concomitantly with replication of the triplex DNA. The results are shown in figure 16. When the

Figure 15. Replication of dA:dT and dA:dT:rU by *E.Coli* DNA polymerase; degradation of the RNA moiety of dA:dT:rU by nuclease contaminating the DNA polymerase. The DNA polymerase reaction mixtures contained 50 mM tris pH 8, 15 mM $MgCl_2$, 2 mM TTP, 2 mM ^{3}H-dATP, 54.6 mμmoles/ml dA:dT or 81.9 mμmoles/ml dA:dT:rU, and 40 μg/ml *E.Coli* DNA polymerase fraction 7. The nuclease assay contained 50 mM tris pH 8, 15 mM $MgCl_2$, 2 mM TTP, 2 mM dATP, 81.9 mμmoles/ml ^{14}C-dA:dT:rU*, and 40 μg/ml *E.Coli* DNA polymerase fraction 7. Both assays were done at 37°.

○——○ nuclease assay; ■——■ dA:dT replication;

▲——▲ dA:dT:rU replication

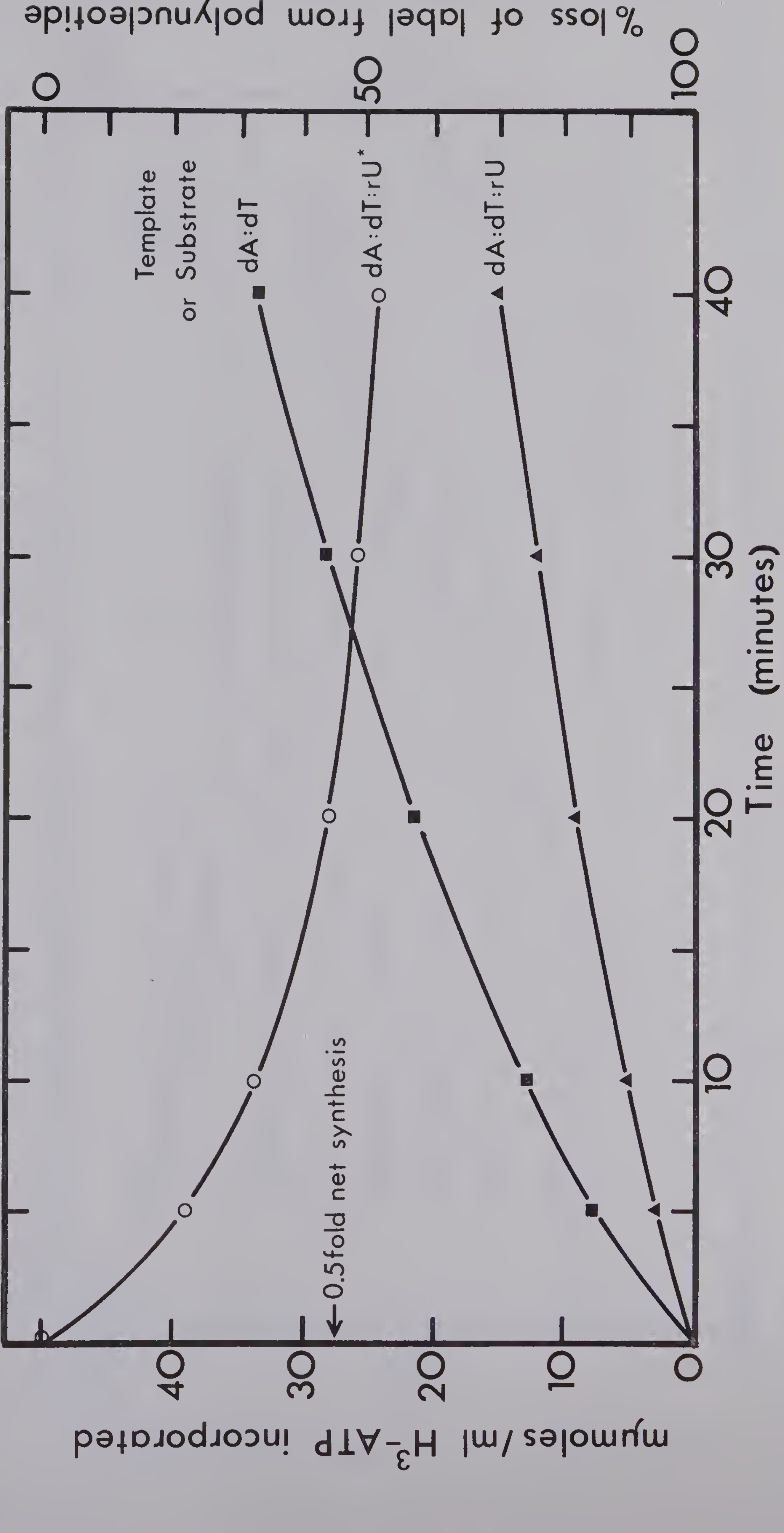
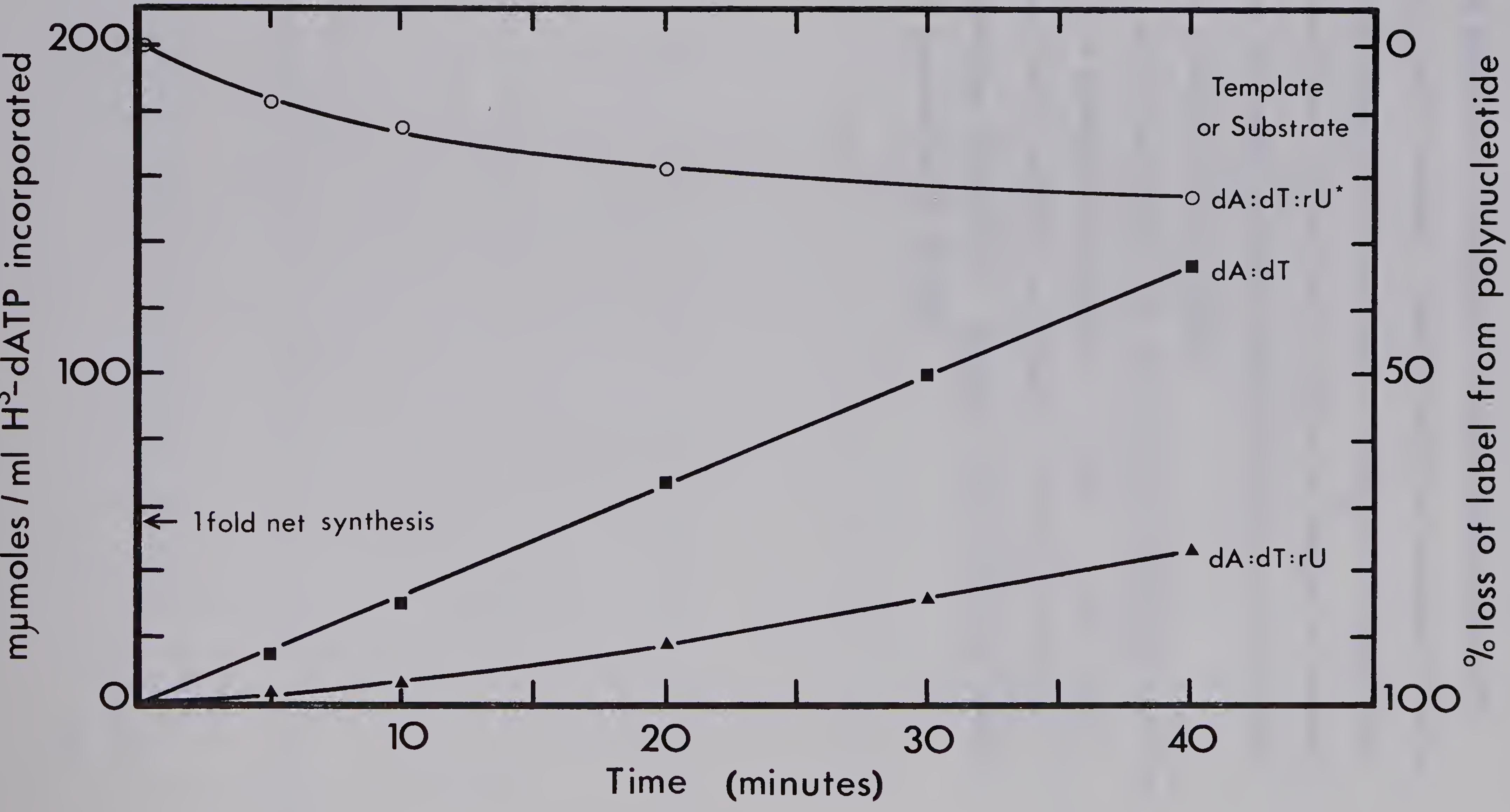

% loss of label from polynucleotide
0
50
100
Template
or Substrate
dA:dT
dA:dT:rU*
dA:dT:rU
←0.5fold net synthesis
mμmoles/ml H3-ATP incorporated
40
30
20
10
0
10
20
30
40
Time (minutes)

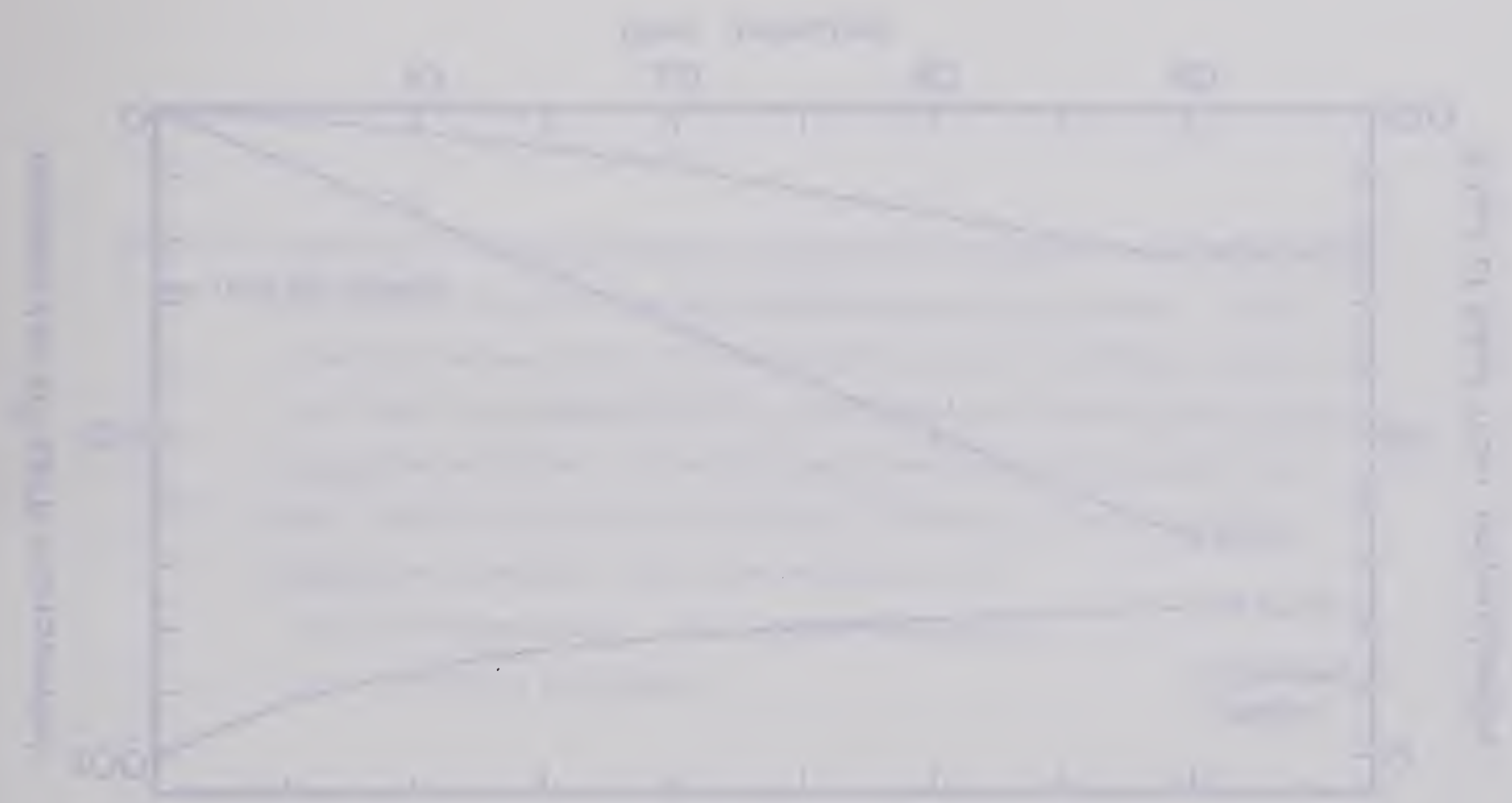
Figure 16. Replication of dA:dT and dA:dT:rU by *M.luteus* DNA polymerase; degradation of the RNA moiety of dA:dT:rU by nuclease contaminating the DNA polymerase. The DNA polymerase reaction mixtures contained 50 mM tris pH 8, 15 mM $MgCl_2$, 2 mM TTP, 2 mM ^3H-dATP, 54.6 mμmoles/ml dA:dT or 81.9 mμmoles/ml dA:dT:rU, and 40 units/ml *M.luteus* DNA polymerase. The nuclease assay contained 50 mM tris pH 8, 15 mM $MgCl_2$, 2 mM TTP, 2 mM dATP, 81.9 mμmoles/ml ^{14}C-dA:dT:rU*, and 40 units/ml *M.luteus* DNA polymerase. Both assays were done at 37°.

○——○ nuclease assay; ■——■ dA:dT replication;

▲——▲ dA:dT:rU replication



200
100
0
mμmoles / ml H3-dATP incorporated
← 1fold net synthesis
10
20
30
40
Time (minutes)
0
50
100
% loss of label from polynucleotide
Template
or Substrate
○ dA:dT:rU*
■ dA:dT
▲ dA:dT:rU


0
100
100
0

triplex was supplied as a template, initially there was an 80% inhibition of dA:dT synthesis, compared with the synthesis observed when dA:dT was supplied as the template. This per cent inhibition decreased with time to approximately 65% at 40 minutes of synthesis. Perhaps this decreasing per cent inhibition was due to the gradual degradation of poly rU in the triplex.

When poly rG was added to an assay mixture containing dA:dT as template, at a phosphate molar ratio of 2 dA:dT : 1 poly rG, no inhibition of dA:dT synthesis was observed, relative to an assay mixture containing dA:dT alone. Similarly, poly rU did not inhibit the rate of replication of dAT:dAT.

Degradation of dA:dT and dA:dT:rU by Pancreatic Ribonuclease and DNase I

1) Pancreatic RNase:

The results of pancreatic RNase digestion of ^{14}C-labeled poly rU and ^{14}C-dA:dT:rU* are shown in figure 17. The initial rate of degradation of poly rU in the triplex was 30% slower than the initial degradation rate of poly rU alone. The addition of dTC:dGA to a reaction mixture containing ^{14}C-poly rU did not inhibit the degradation of poly rU. Thus the presence of the DNA in the triplex offered some protection to the RNA against RNase attack, though not complete protection under the conditions studied. As shown in figure 17, the RNase contained no DNase activity detectable by this method of assay.

2) DNase I:

The results of DNase I degradation of ^{14}C-labeled dA*:dT* and dA*:dT*:rU are shown in figure 18. The initial rate of degradation of the DNA in the triplex was about 30% slower than that of dA:dT. That the DNase I preparation used did not contain RNase activity was confirmed by incubating the substrate ^{14}C-dA:dT:rU* with DNase I and checking for loss of acid insoluble label with time. These results are also shown in figure 18. Addition of poly rAU to an assay mixture containing ^{14}C-dA*:dT* as substrate did not inhibit degradation of the DNA.

In the presence of Mg^{+2} the mechanism of DNase I action is a so-called double hit mechanism. Each encounter of the enzyme with DNA results in cleavage of only one of the two strands (27). Thus

Figure 17. Pancreatic RNase degradation of poly rU and dA:dT:rU. The reaction mixtures contained 50 mM Na phosphate pH 7.3, 10 mM $MgCl_2$, 55 mμmoles/ml ^{14}C-poly rU or 83 mμmoles/ml ^{14}C-dA:dT:rU*, and 25 μg/ml pancreatic RNase. Assays were done at 37°.

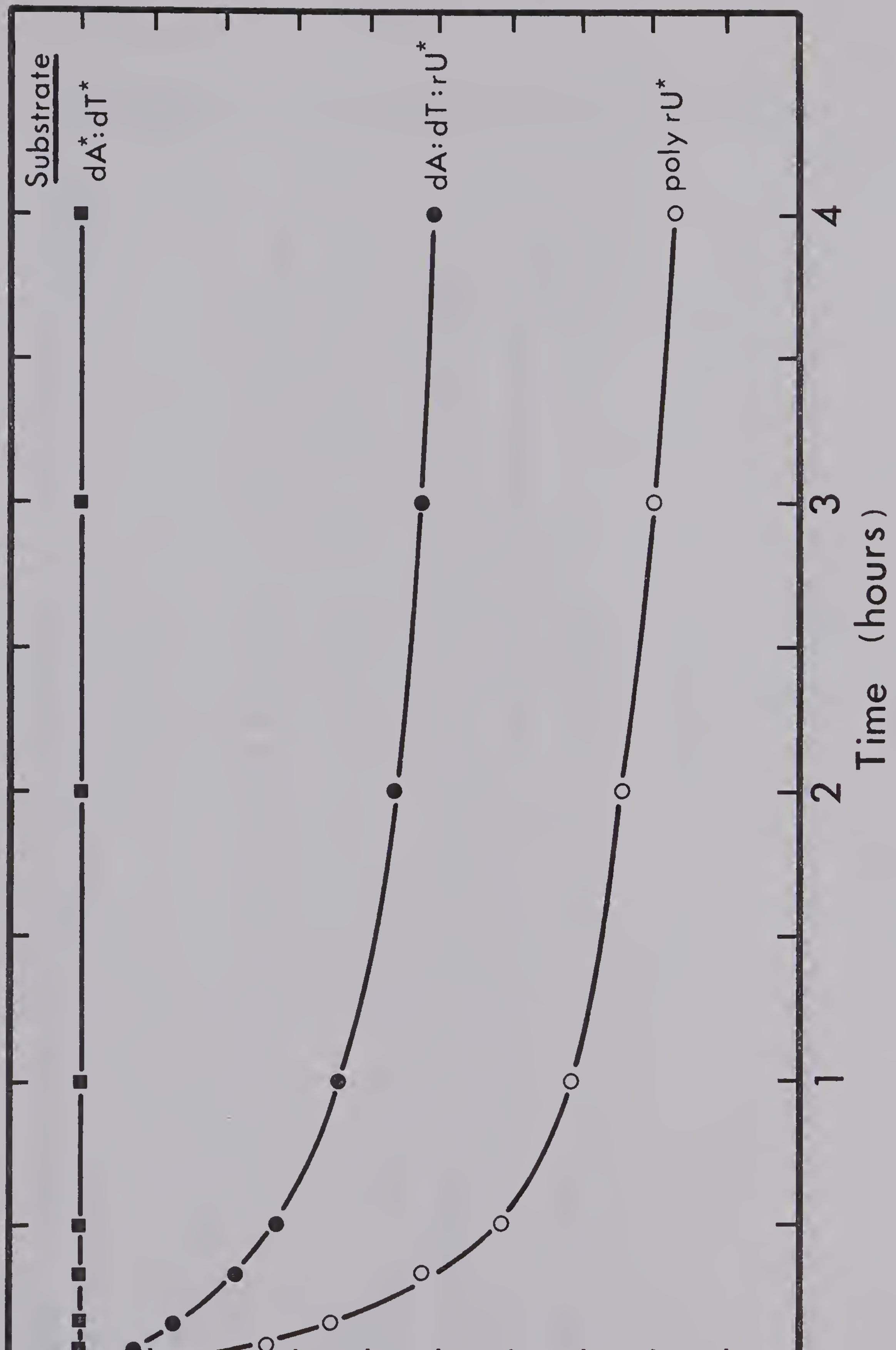
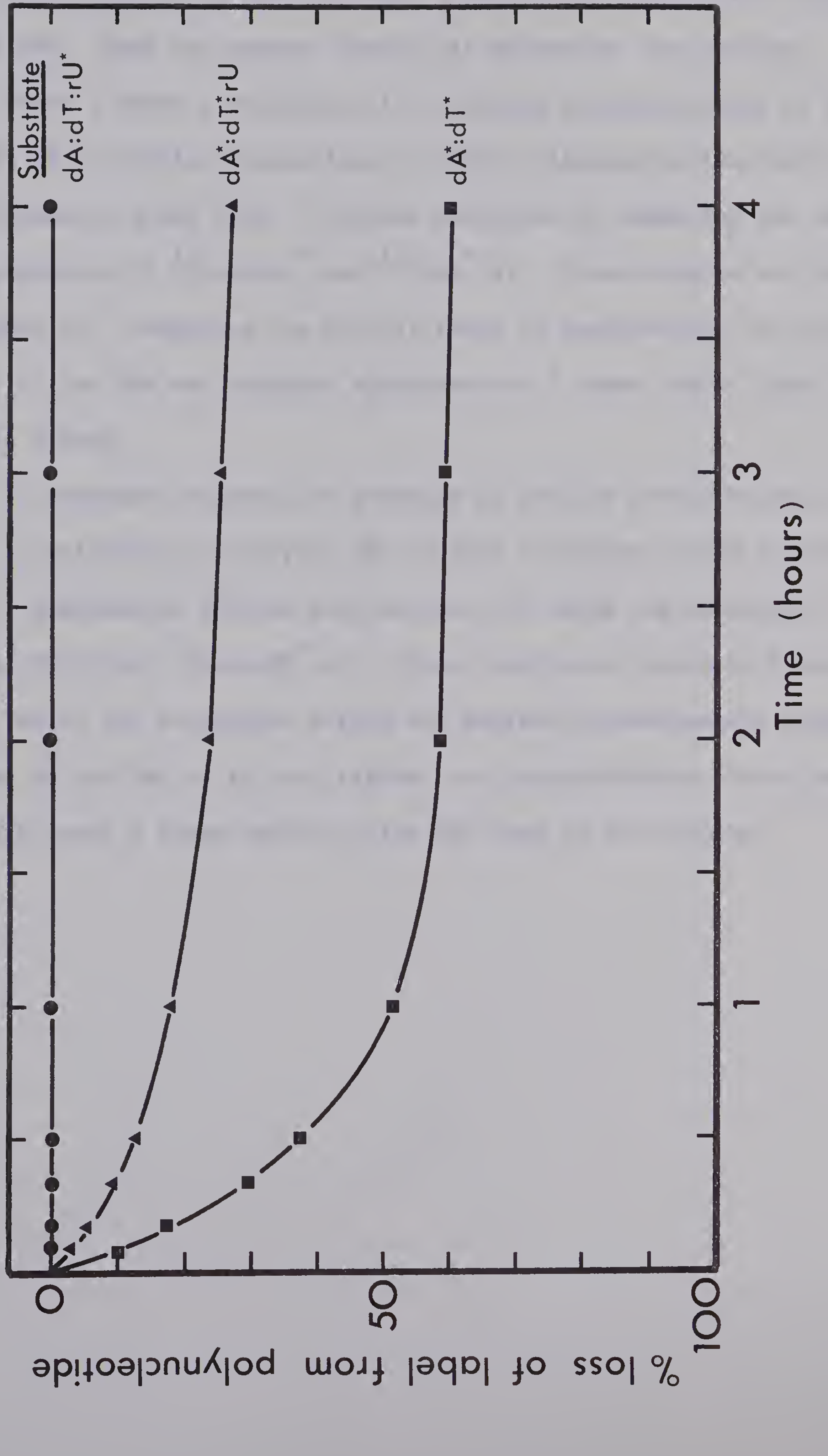

Substrate
dA*:dT*
dA:dT:rU*
poly rU*
% loss of label from polynucleotide
0
50
100
Time (hours)
1
2
3
4

Figure 18. DNase I degradation of ^{14}C-dA*:dT* and ^{14}C-dA*:dT*:rU. The reaction mixtures contained 50 mM Na phosphate pH 7.3, 10 mM MgCl$_2$, 90 mμmoles/ml ^{14}C-dA*:dT* or 135 mμmoles/ml ^{14}C-dA*:dT*:rU or 135 mμmoles/ml ^{14}C-dA:dT:rU*, and 5 μg/ml DNase I. Assays were done at 37^{o}.

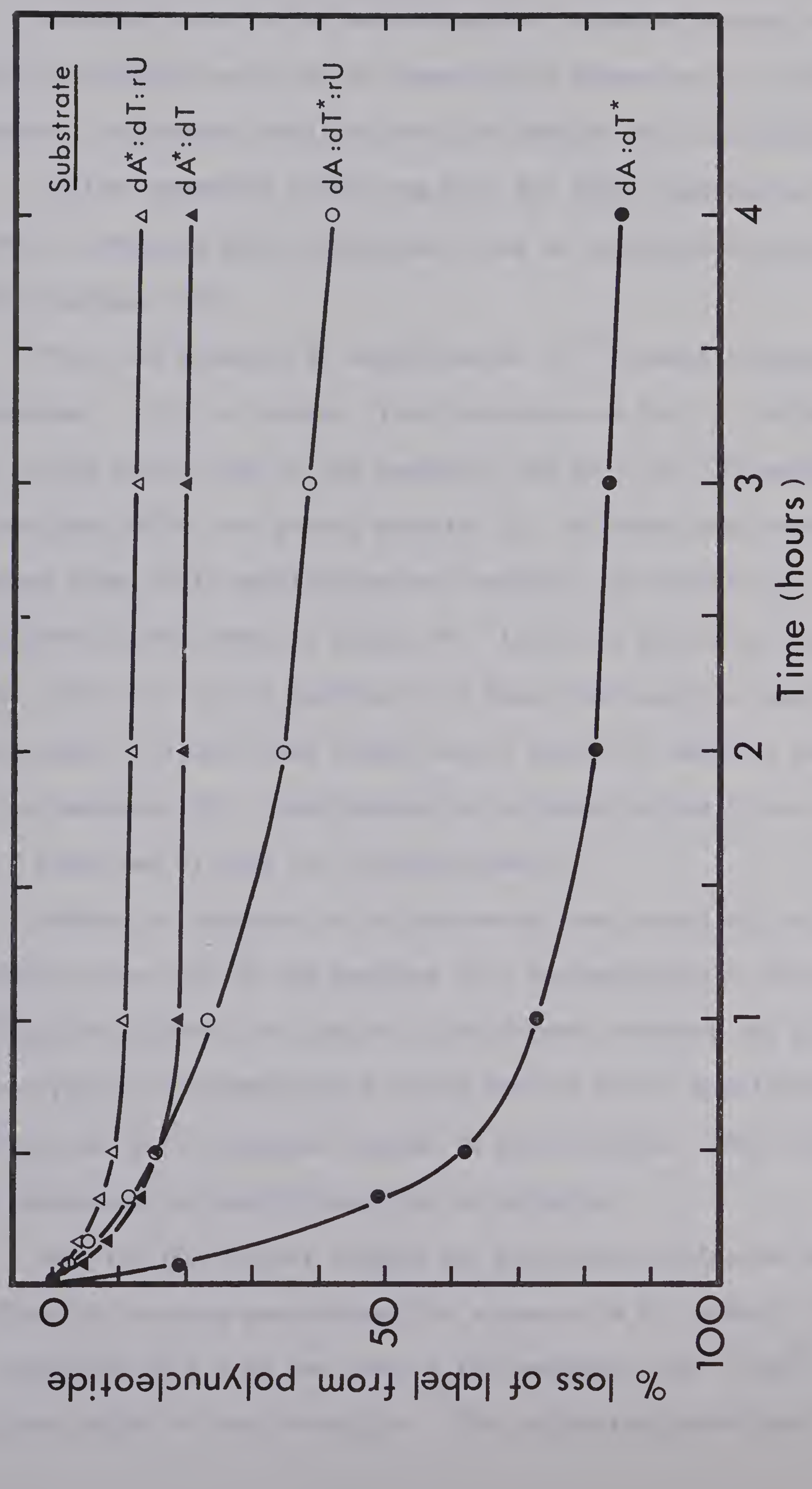

Substrate
dA:dT:rU*
dA*:dT*:rU
dA*:dT*
% loss of label from polynucleotide
0
50
100
Time (hours)
1
2
3
4

a second cleavage must occur in the other strand in the vicinity of the first scission in order to cause a reduction in molecular weight of the DNA. Thus the phrase 'double hit mechanism' has evolved.

DNase I shows a preference for cleaving polynucleotides on the 5' side of pyrimidine nucleotides, to yield oligonucleotides with a 5' phosphate group (18). This was confirmed by comparing the rates of degradation of ^{14}C-dA:dT* and ^{14}C-dA*:dT. These results are shown in figure 19. Comparing the initial rates of degradation, the poly T strand of the DNA was degraded approximately 5 times faster than the poly dA strand.

To determine whether the presence of poly rU conferred equal DNase I resistance to the poly dA and poly T strands of the triplex, DNase I degradation studies were carried out using the substrates ^{14}C-dA*:dT:rU and ^{14}C-dA:dT*:rU. These results are shown in figure 19 also. While the polypurine strand was degraded approximately twice as fast in the DNA as in the triplex, the polypyrimidine strand was degraded about 6 times faster in the DNA than in the triplex.

Figure 19. DNase I degradation of ^{14}C-dA*:dT, dA:dT*, dA*:dT:rU, and dA:dT*:rU. Reaction mixtures contained 50 mM Na phosphate pH 7.3, 10 mM MgCl$_2$, 90 mμmoles/ml ^{14}C-DNA or 135 mμmoles/ml ^{14}C-triplex, and 5 μg/ml DNase I. Assays were done at 37°.


Substrate
dA*:dT:rU
dA*:dT
dA:dT*:rU
dA:dT*
0
50
100
1
2
3
4
Time (hours)
% loss of label from polynucleotide


0
50
100

Spermine Binding to dA:dT and dA:dT:rU

Although quantitative measurements of spermine binding to either dA:dT or dA:dT:rU could not be reproducibly determined by equilibrium dialysis, nonetheless qualitatively the results were informative.

Sodium cacodylate buffer was used for these experiments because spermine complexes with citrate and forms an insoluble precipitate with phosphate (29).

First the kinetics of equilibration of ^{14}C-labeled spermine were determined. 35 μl of buffer (5 mM cacodylate pH 6.8, 0.1 M NaCl) was placed on one side of the membrane, and 35 μl of ^{14}C-spermine in the same buffer was placed opposite it. Aliquots were removed at various times until equilibrium was reached. The kinetics of equilibration are shown in figure 20. As can be seen from the graph, there was a net loss of spermine with time, plateauing as equilibrium was reached. Likely these losses were a result of spermine binding to the membrane (30). Equilibrium was attained within 5 hours at 24°, but 7 hours was allowed for each experiment.

Binding of spermine to polynucleotide was tested by placing the polymer on one side of the membrane at a concentration of 230 mμmoles/ml. ^{14}C-labeled spermine was placed in the chamber opposite the first. The concentration of spermine used varied from 30 to 150 mμmoles/ml. Caution had to be exercised because at high spermine : DNA ratios, the DNA aggregated and precipitated out of solution.

That the DNA did not contain any dialyzable nucleotide was confirmed by placing non-radioactive spermine in 0.1 M NaCl, 5 mM Na cacodylate pH 6.8 on one side of the membrane, and ^{14}C-dA*:dT* in the same buffer on the other side. The radioactive dA:dT had been

Figure 20. Kinetics of equilibration of spermine at 24°. In equilibrium dialysis cells, 35 μl of ^{14}C-spermine in 5 mM Na cacodylate pH 6.8, 0.1 M NaCl was placed on one side of the membrane, and 35 μl of the same buffer was placed opposite it. Aliquots were removed at various times.

△———△ chamber containing buffer;

□———□ chamber containing ^{14}C-spermine

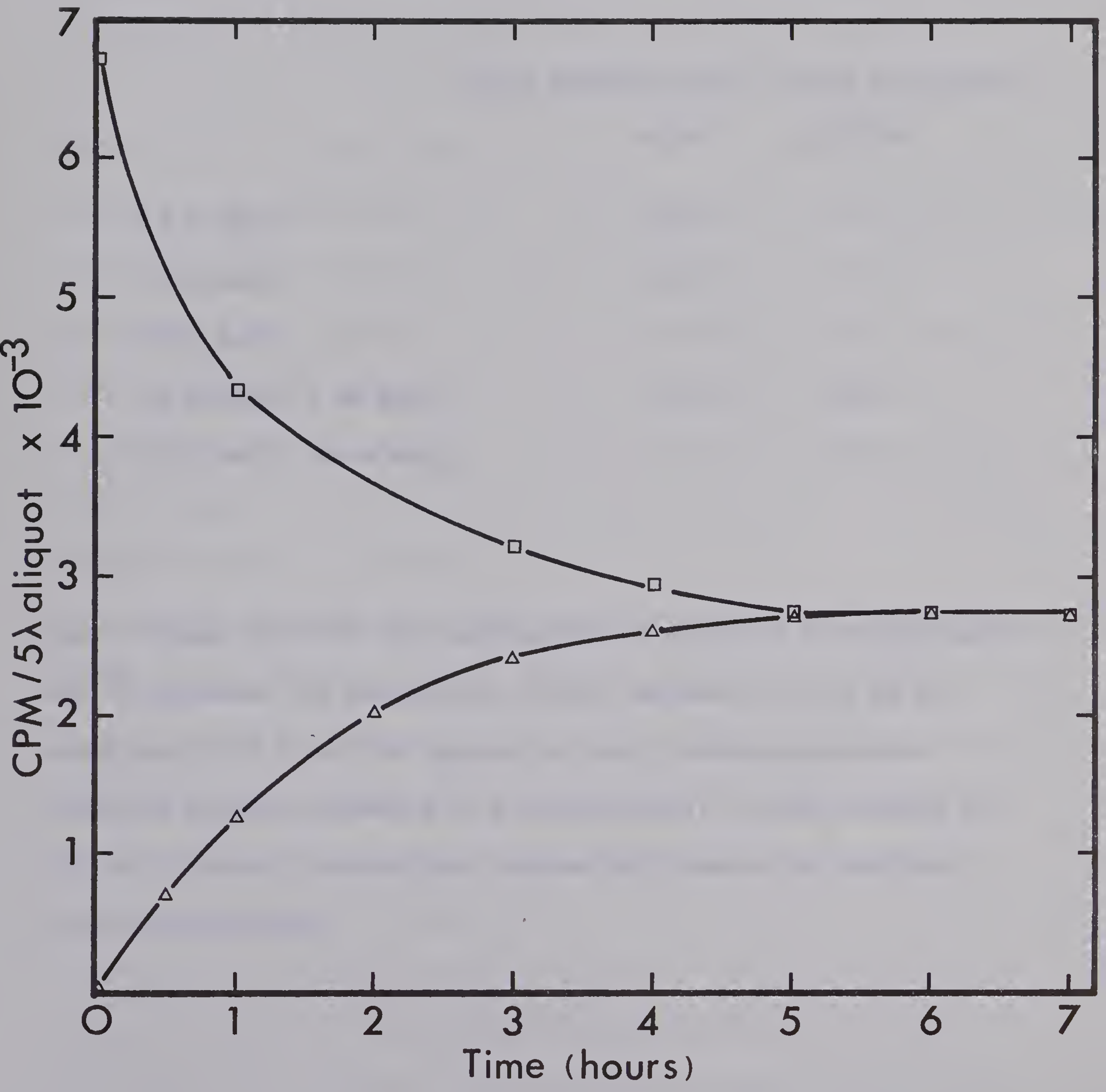

7
6
5
4
3
2
1
CPM / 5λ aliquot × 10⁻³
O
1
2
3
4
5
6
7
Time (hours)

TABLE 1

EQUILIBRIUM DIALYSIS OF ^{14}C-SPERMINE AGAINST dA:dT AND dA:dT:rU UNDER VARIOUS SALT CONDITIONS

	Moles spermine bound / mole nucleotide P	
	dA:dT	dA:dT:rU
0.1 M NaCl	.066	-
0.3 M NaCl	.031	-
0.5 M NaCl	0	0
0.1 M NaCl, 2 mM $MgCl_2$	.039	.045
0.1 M NaCl, 50 mM $MgCl_2$	0	0

Equal volumes of dA:dT (230 mμmoles/ml) or dA:dT:rU (230 mμmoles/ml) and ^{14}C-spermine (75 mμmoles/ml, 26,000 cpm/mμmole) in 5 mM Na cacodylate pH 6.8 and the appropriate salt concentration were placed in opposing chambers of a dialysis cell. After shaking at 24° for 7 hours, aliquots were removed and counted in omnifluor scintillation fluid.

prepared and isolated by the same procedure as the non-radioactive dA:dT. After shaking at 24° for 7 hours, aliquots taken from each chamber were counted. No transfer of radioactive material across the membrane could be detected.

Binding of spermine to dA:dT could be detected in the presence of 5 mM Na cacodylate pH 6.8, 0.1 M NaCl. When 2 mM $MgCl_2$ was added to the buffer, spermine binding to dA:dT was reduced, but still readily detectable. When the NaCl concentration was raised from 0.1 M to 0.3 M, spermine binding was also reduced, and at 0.5 M NaCl binding was no longer detectable. Similarly, when the $MgCl_2$ concentration was raised from 2 mM to 50 mM, spermine binding was no longer detectable. This data is shown in table 1. These results are not unexpected since spermine acts as a cation, having a net charge of four at pH 6.8 (29). Thus as the ionic strength was raised, competition for spermine binding sites became more and more successful, until finally spermine binding was abolished.

The relative levels of $MgCl_2$ and NaCl required to prevent spermine binding suggest that the binding affinity of spermine for DNA is much greater than that of monovalent cations for DNA. In fact, the results of Felsenfeld and Huang (31) show that polyamines are a little less strongly bound to rA:rU than divalent cations, but the affinities are of the same order of magnitude.

Spermine was shown to bind to dA:dT:rU somewhat stronger than to dA:dT. Similar to the results described above, when the $MgCl_2$ concentration was raised to 50 mM, spermine binding to dA:dT:rU was no longer detectable. No binding of spermine to the triplex was observed in the presence of 0.5 M NaCl. These results are also shown in table 1.

That spermine at these concentrations did not cause dissociation of the triplex was confirmed spectrophotometrically.

DISCUSSION

It has been shown that on combining dA:dT and poly rU in a phosphate molar ratio of 2:1, 14% hypochromicity is observed at 260 nm, when compared with a theoretical absorbance for non-interacting polynucleotides.

Mixing curves performed under a variety of conditions gave two straight lines intersecting at 33% poly rU. Although this in itself does not distinguish between dA:dT:rU and a mixture of dA:$(dT)_2$ and dA:$(rU)_2$, it has been shown previously that only a single multi-stranded species is formed (21).

In the absence of $MgCl_2$, the KCl titration of a solution containing dA:dT and poly rU indicates that interaction between the two polymers occurs gradually over a broad range of KCl concentrations. This lack of cooperativity suggests that intermediate, graded levels of organization of the RNA with the DNA can occur.

In the absence of KCl, the addition of $MgCl_2$ to a solution containing dA:dT and poly rU results in conversion to a triplex over a relatively narrow range of $MgCl_2$ concentrations. This cooperativity implies an all or none phenomenon for triplex formation in the presence of $MgCl_2$.

Both $MgCl_2$ and KCl titration of dA:dT and poly rU were independent of polynucleotide concentration. These results are in contrast to those of Felsenfeld and Huang, who showed that NaCl titration of a solution containing poly rA and poly rU was independent of polynucleotide concentration, while divalent ion titration showed a dependence on polynucleotide concentration (31). The interaction

of poly rA and poly rU required one equivalent of divalent ion / mole of phosphate to go to completion. The data of Felsenfeld and Huang indicate that the NaCl concentration necessary for poly rA and poly rU interaction is approximately 30 times less than the concentration we found necessary for dA:dT and poly rU interaction. This would imply that either 1) for triplex formation a greater charge barrier must be overcome than for duplex formation, or 2) a more ionic environment is necessary for stabilization of a triplex than for RNA duplex stabilization.

The alkaline titration curve for dA:dT:rU indicates that the poly rU strand is melted off first, followed by melting of dA:dT at higher pH. These results are similar to the thermal melting studies of Riley et al. (21). On alkali titration of the triplex from pH 7.3, melting of the RNA occurs between pH 8.2 and 9.0. It is not a cooperative phenomenon as is the melting of DNA. Although the pK_a's of uridine and thymidine are very similar (9.5 and 9.8 respectively), the ionization of these bases in the triplex occurs at very different pH's. Thus the poly T strand appears to be in a more thermodynamically stable configuration than the poly rU strand in the triplex.

Acid titration of the triplex indicates that its entire melting occurs in one step. On triplex formation the poly rU strand is believed to complex with dA:dT by hydrogen bonding with the adenine bases at the N6 amino groups and N7 imino groups. Thus on protonation of the adenine moiety, both the Watson-Crick base pairing with the poly T and the pairing with the poly rU strand would be destroyed at the same time.

Transcription and replication studies of the triplex indicate that

it can be both transcribed and replicated to some extent by the enzyme preparations used. Relevance of these results to the in vivo situation is obviously limited by the nature of the assays. In vivo, proteins probably play a large role in gene repression. For example, it has been shown by Bonner et al. that histones inhibit the in vivo and in vitro transcription of DNA (32). However, our results suggest that triplexes may play a part in gene expression and duplication.

Bekhor et al. have shown that for specific reconstitution of eukaryotic chromatin, the participation of chromosomal RNA is required (33). 'Specific' reconstitution here implies the production of similar RNA sequences on transcription of the chromatin before dissociation and after reconstitution. Thus specificity of gene repression may result from specificity of triplex formation.

The degradation of DNA and RNA in the triplex by DNase I and pancreatic RNase occurred at slower rates than in the uncomplexed forms. These results may have some relevance to the in vivo situation, whereby a triplex could confer resistance to the DNA and/or RNA against nuclease attack. However, again it must be pointed out that proteins and other smaller moieties are probably also involved in such a protective mechanism in vivo. For example, it has been shown that spermine protects ribosomes against digestion by trypsin and ribonuclease (33).

During the course of the enzyme degradation studies it was hoped that the triplex might be totally insensitive to either DNase I or pancreatic RNase activity. If this were the case, then a tool would be made available for isolation of triplexes from organisms. Conditions may exist under which triplexes are completely resistant to nuclease

degradation. Further studies are necessary in order to investigate this possibility.

Equilibrium dialysis studies indicate that spermine binds to the triplex dA:dT:rU. Suwalsky et al. have shown by X-ray crystallography that spermine binds to DNA in the minor groove (34). They have shown with molecular models that it is possible for all 4 basic groups of a spermine molecule to make ionic and hydrogen bonds with phosphate groups on one molecule of DNA. In this way the spermine molecules would lie across the minor groove of DNA, and serve to bind its two strands together. This would explain the elevated T_m of DNA in the presence of polyamines.

It has been postulated that in a DNA-RNA triplex the RNA winds around the DNA in the major groove. The observed binding of spermine to the triplex confirms this hypothesis. In fact, the binding of spermine to DNA could be used as a probe for investigating the type of interaction involved between DNA and other molecules, for example antibiotics or proteins. A lack of spermine binding to DNA in the presence of a particular antibiotic or protein fraction would suggest a binding site for it in the minor groove of DNA.

These studies have revealed a number of interesting properties of the triplex dA:dT:rU. If triplexes are to be isolated from organisms, suitable salt conditions must be maintained throughout the isolation procedure. Use of SDS as a protein denaturant during triplex isolation would not disrupt the polynucleotide complexes. pH conditions would have to be controlled carefully to avoid triplex melting.

Our data supports the hypothesis that triplexes may play a role in gene duplication and transcription in vivo. Accumulated evidence suggests that triplexes may exist at promoter sites, thereby

regulating the initiation of gene transcription by RNA polymerase. One might envisage subtle changes in salt and/or pH conditions at the triplex site which would cause dissociation of the triplex, and perhaps allow RNA polymerase to initiate RNA synthesis. These ionic strength and/or pH changes may be mediated by conformational changes occurring in nearby proteins.

Repressors for both the lac operon and λ genome have been isolated (35, 36). These repressors were reported to be proteins; no nucleic acid component was detected. The lac repressor has been shown to inhibit lac transcription in vitro by 80% (37). Although these results tend to negate a role for RNA in gene repression, nonetheless the existence of a variety of mechanisms for gene repression would not be unexpected.

It has been estimated that polyamines are complexed with a large percentage of the DNA in *E.Coli*. The function of these polyamines is as yet uncertain. The fact that spermine binds to triplexes may be significant with respect to polyamine function in the promoter or operator regions of the *E.Coli* chromosome.

Finally, the large differences which exist in the conditions required for dA:dT:rU and dTC:dGA:rU$\overset{+}{\text{C}}$ formation point out that the characteristics of various triplexes may be quite different. This in itself would provide a possible mechanism for regulation of gene expression.

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